Transvection and Enhancer-Promoter Specificity at the *D-Pax2* Locus during *Drosophila* Development

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von
Sabarinadh Chilaka

aus
Indien

Promotionskomitee
Prof. Dr. Markus Noll
(Vorsitz und Leitung der Dissertation)
Prof. Dr. Konrad Basler

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Summary

The *Drosophila Pax2* (*D-Pax2*) gene encodes a paired-domain containing transcription factor, most closely related to the vertebrate *Pax2*/*5*/*8* subfamily. Like its mammalian counterpart, *D-Pax2* plays an important role in the development of several sensory organs. During eye development, it is required for the proper specification and differentiation of cone and primary pigment cells. The *spa* enhancer regulating *D-Pax2* transcription in the developing eyes has been mapped previously to the 4th intron. The D-Pax2 protein also plays a vital role in the developing peripheral nervous system (PNS), where it regulates the proper specification and differentiation of shaft and sheath cells of mechanosensory bristles. The *sv* enhancer, regulating *D-Pax2* transcription in the PNS, is located upstream of the *D-Pax2* transcription initiation site. In my thesis, I have addressed two important aspects of the transcriptional regulation of *D-Pax2* as well as its function in the developing central nervous system (CNS).

An intriguing property of enhancers is that they specifically target and activate transcription from their cognate promoter. The regulatory mechanisms responsible for enhancer-promoter specificity are not well understood. Using the complex *D-Pax2* locus, which includes several tissue-specific enhancers and two different promoters, I was able to investigate this question within a single gene since the enhancers act only through one of the two promoters. The *sv* enhancer and the *spa* enhancer specifically activate transcription from the PNS promoter, whereas the CNS enhancer specifically activates transcription from the CNS promoter. The specificity between these enhancers and their cognate promoters is achieved by promoter-enhancer compatibility (or incompatibility) rather than promoter-competition or an insulator element. It is further shown that promoters rather than enhancers are controlling this enhancer-promoter compatibility, since the *sv* and CNS enhancers, which specifically activate their cognate promoters, can also activate the P-element promoter.

Initially, enhancers were thought to activate transcription only in *cis*. However, the discovery and genetic studies of a phenomenon called transvection illustrated that enhancers can also activate transcription in *trans* from the promoter located on the homologous chromosome. I report here on the phenomenon of transvection at the *D-Pax2* locus. By genetic tests I could demonstrate that the *spa* enhancer exhibits transvection by activating the PNS promoter on

the other *D-Pax2* allele. However a second enhancer of this locus, the *sv* enhancer, activating the same promoter in *cis* does not display transvection. Thus, two enhancers of a single gene locus, activating a single cognate promoter, differ in exhibiting transvection. These results demonstrated for the first time that not all enhancers have the ability to activate in *trans* and that the property of an enhancer to activate in *trans* depends on the enhancer rather than the promoter.

Like its vertebrate homolog, *D-Pax2* is also expressed in the developing CNS. Large *D-Pax2* deletion alleles were generated that, lacking both promoters, are complete null alleles for all known *D-Pax2* functions. Analysis of these alleles showed that *D-Pax2* plays an important role in CNS development. Loss of *D-Pax2* function in the CNS is lethal during the second larval instar. A crucial *cis*-regulatory region responsible for *D-Pax2* expression in the CNS was mapped to the highly conserved 6^{th} intron. *D-Pax2* transcription under control of this intron rescues many but not all CNS null animals to adulthood. This suggests that additional CNS enhancer elements are required for complete rescue.

Zusammenfassung

Das *Drosophila Pax2* Gen (*D-Pax2*) kodiert für einen Paired-Domänen Transkriptionsfaktor, welcher der *Pax2/5/8* Unterfamilie bei Wirbeltieren sehr ähnlich ist. Wie sein Pendant in Säugetieren spielt *D-Pax2* eine wichtige Rolle bei der Entwicklung mehrerer Sinnesorgane. Während der Augenentwicklung ist es für die korrekte Spezifikation und Differenzierung der Kegel- und primären Pigmentzellen erforderlich. Schon früher wurde der *spa*-Enhancer, der die *D-Pax2* Expression in den sich entwickelnden Augen reguliert, im vierten Intron kartiert. Das D-Pax2 Protein spielt auch eine entscheidende Rolle in der Entwicklung des peripheren Nervensystems (PNS), wo es die richtige Spezifikation und Differenzierung der Schaft- und Mantelzellen der mechanosensorischen Tastborsten steuert. Der *sv*-Enhancer, der die *D-Pax2* Transkription im PNS steuert, befindet sich oberhalb des *D-Pax2* Transkriptionstarts. In meiner Dissertation habe ich zwei wichtige Aspekte der Transkriptionsregulation von *D-Pax2* und die Funktion von *D-Pax2* in der Entwicklung des zentralen Nervensystems (ZNS) untersucht.

Eine interessante Eigenschaft von Enhancern ist, dass sie gezielt ausschliesslich ihren eigenen Promotor aktivieren. Die für diese Enhancer-Promotor Spezifität verantwortlichen Mechanismen sind nicht gut verstanden. Mit dem komplexen *D-Pax2* Lokus, der mehrere gewebespezifische Enhancer und zwei verschiedene Promotoren hat, konnte ich diese Frage mit einem einzelnen Gen untersuchen, da die Enhancer jeweils nur auf einen der Promotoren wirken. Der *sv*-Enhancer und der *spa*-Enhancer aktivieren spezifisch die Transkription des PNS-Promotors, hingegen der ZNS-Enhancer die Transkription des ZNS-Promotors. Die Spezifität zwischen diesen Enhancern und ihren eigenen Promotoren wird durch Promotor-Enhancer-Kompatibilität (oder Inkompatibilität) zwischen diesen Enhancern und Promotoren, nicht jedoch durch Promotor-Kompetition oder ein Insulator DNA-Element gesteuert. Es wurde ferner gezeigt, dass die Promotoren und nicht die Enhancer entscheidend sind für die Kontrolle dieser Enhancer-Promotor-Kompatibilität, da sowohl der *sv*- als auch der ZNS-Enhancer, die ihren eigenen Promotor spezifisch aktivieren, auch den P-Element-Promotor aktivieren können.

Früher wurde angenommen, dass Enhancer die Transkription nur in *cis* aktivieren können. Aber die Entdeckung und Untersuchungen des “Transvektion” Phänomens haben gezeigt,

dass Enhancer auch die Transkription in *trans* vom Promotor, der sich auf dem homologen Chromosom befindet, aktivieren können. Ich konnte zeigen, dass Transvection auch beim *D-Pax2* Lokus vorkommt. Mit genetischen Tests fand ich heraus, dass der *spa*-Enhacer zu Transvektion fähig ist, da er die Transkription des PNS-Promotors auf dem anderen *D-Pax2* Allel aktivieren kann. Allerdings zeigt ein zweiter Enhancer dieses Lokus, der *sv*-Enhancer, der denselben Promotor in *cis* aktivieren kann, keine Transvection. So können sich zwei Enhancer eines Gens, die den gleichen Promotor aktivieren, in ihrer Fahigkeit zur Transvektion unterscheiden. Insgesamt konnte zum ersten Mal gezeigt werden, dass nicht alle Enhancer eines Gens in der Lage sind, in *trans* zu aktivieren und dass die Eigenschaft eines Enhancers in *trans* zu aktivieren vom Enhancer und nicht vom Promotor abhängt.

Wie sein homologes Gen in Wirbeltieren wird *D-Pax2* auch während der Entwicklung des ZNS exprimiert. Allele mit grossen *D-Pax2* Deletionen wurden isoliert, die, da beide Promotoren entfernt wurden, Null-Allele für alle bekannten *D-Pax2* Funktionen sind. Die Analyse dieser Allele hat gezeigt, dass *D-Pax2* eine wichtige Rolle bei der Entwicklung des ZNS spielt. Der Verlust der *D-Pax2* Funktion im ZNS ist im zweiten Larvenstadium letal. Eine für die *D-Pax2* Expression im ZNS verantwortliche *cis*-regulatorische Region konnte dem hoch konservierten sechsten Intron zugeordnet werden. *D-Pax2* Transkription unter der Kontrolle dieses Introns rettet viele, aber nicht alle ZNS-Null Tiere bis zum Erwachsenenalter. Dies legt den Schluss nahe, dass für eine vollständige Rettung zusätzliche ZNS Enhancer-Elemente erforderlich sind.

Chapter 1

General Introduction

Pax genes, originally discovered in Noll's lab on the basis of the gene network concept (Frigerio et al., 1986; Bopp et al., 1986; Noll, 1993), encode a family of transcription factors comprising a highly conserved DNA binding paired domain. These genes play a significant role in various developmental processes of vertebrates and invertebrates (Frigerio et al., 1986; Bopp et al., 1986; Noll, 1993). *D-Pax2*, also known as *shaven* (*sv*) or *sparkling* (*spa*), is the *Drosophila* homolog of the vertebrate *Pax-2/5/8* gene subfamily (Fu and Noll, 1997; Fu et al., 1998). Like its mammalian counterpart, it plays an important role in the development of several sensory organs (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999; Shi, 2001). *D-Pax2* is expressed in the developing peripheral and central nervous system (PNS and CNS) and in developing eyes (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999; Shi, 2001). For some time, the *sv* and *spa* mutant alleles were thought to represent two neighboring genes, but it was later shown by our lab that these are mutations in two different tissue-specific enhancers of *D-Pax2*, the *sv* or PNS enhancer and the *spa* or eye enhancer (Fu et al., 1998).

Role of *D-Pax2* in the *Drosophila* PNS development

The mechanosensory bristle is an external sensory organ of the adult *Drosophila* and comprised of four cell types, a tormogen (socket cell), trichogen (shaft cell), thecogen (sheath cell), and neuron. These arise from a single sensory organ precursor cell (SOP) through a stereotypical series of asymmetric divisions (Fig. 1) (for review, see Posakony, 1994). SOPs are selected from a small group of cells, the proneural cluster, endowed with SOP fate potential by the expression of the proneural genes of the achaete-scute complex which encode bHLH transcription factors. After specification of the SOP, all other cells of the proneural cluster are inhibited from becoming SOPs through a process called lateral inhibition (Ghysen et al., 1993). The primary SOP cell, PI, divides asymmetrically to produce two sibling cells, PIIa and PIIb (Fig. 1B). The PIIa cell divides to produce the shaft and socket cell, while the PIIb cell divides to give rise to a PIIIb and a glial cell. The PIIIb cell again divides asymmetrically to generate a neuron and a sheath cell (Fig. 1B) (Gho et al., 1999; Reddy and

Rodrigues, 1999). Recently, it has been shown that the glial cell generated in this lineage undergoes apoptosis (Fichelson and Gho, 2003).

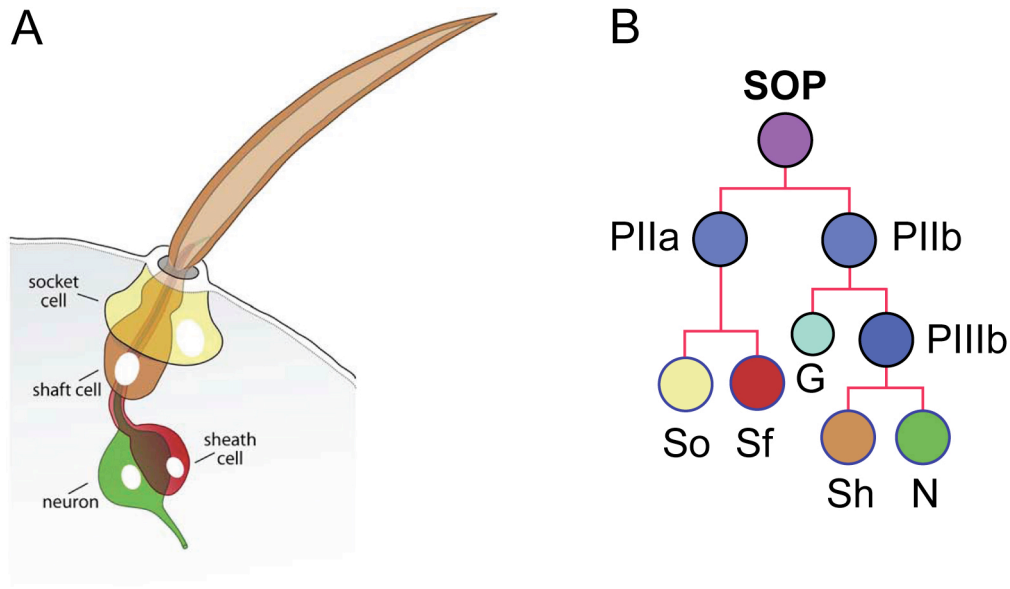

Fig. 1. Structure and lineage of adult mechanosensory bristle.
(A) Structure of adult mechanosensory bristles (adapted from Fabre et al., 2008). The mature sensory organ comprises four cells, a shaft cell (brown), socket cell (yellow), a monodendritic sensory neuron (green) and a sheath cell (red). **(B)** Cell lineage of mechanosensory bristle, with terminally differentiated socket (So), shaft (Sf), glial (G), sheath (Sh) cell, and neuron (N).

D-Pax2 plays a vital role in the developing larval PNS and adult mechanosensory bristles. In the latter it is required for the proper specification and differentiation of shaft and sheath cells (Fu et al., 1998; Kavaler et al., 1999). It is expressed in SOPs and all cells of this lineage (Fu et al., 1998; Kavaler et al., 1999). By mid-pupal stages, it is no longer expressed in the socket and neuron, but continues to be expressed at high levels in sheath and shaft cells (Kavaler et al., 1999). The hypomorphic *sv* alleles, sv^{n} and sv^{de}, exhibit reduced or loss of shaft structures of all bristles, the so-called *shaven* phenotype (Fu et al., 1998). However, $sv^{\Delta 122}$ mutants (Fig. 1 of Chapter 2) that fail to express *D-Pax2* in the PNS and eye die as first instar larvae (Michael Daube and Erich Frei, unpublished), which suggests that *D-Pax2* function in the PNS is vital. The *sv* enhancer responsible for the *D-Pax2* expression in the PNS has been mapped to a region upstream of the PNS promoter (Fu et al., 1998; Kavaler et al., 1999). Further characterization of a minimal *sv* enhancer delimited it to a 760 bp fragment, 1.6 kb

upstream of the initiation site of the PNS promoter (Shi, 2001). Since one copy of a rescue transgene under the control of this region in a null mutant leads to bristles that are still abnormal, some enhancer elements must be located outside this region. The 760 bp minimal *sv* enhancer was further divided into two parts, an early and a late element, which support transcription during the early and late phases of development of both the larval and the adult mechanosensory organs. In addition, the early element is crucial for rescuing the lethality of $sv^{\Delta 122}$ mutants, whereas both the early and late elements are required for the bristle development (Shi, 2001).

Role of *D-Pax2* in eye development

Each *Drosophila* compound eye consists of about 800 unit structures, called ommatidia, that are arranged in a regular symmetrical fashion. Each ommatidium consists of eight photoreceptor (R) cells, R1-R8, four lens-secreting cone cells, and two primary pigment cells, and shares six secondary and three tertiary pigment cells as well as three mechanosensory bristles with neighboring ommatidia (Wolff and Ready, 1993). The compound eye develops from a monolayer of undifferentiated epithelial cells, the eye imaginal disc. During the third larval instar, pattern formation begins as a wave of morphogenesis moving from posterior to anterior across the disc. This wave is marked by a depression in the surface of the disc, the morphogenetic furrow, behind which small and precisely distributed cell clusters differentiate into photoreceptor neurons (Greenwald and Rubin, 1992; Wolff and Ready, 1993). Initially, R8 is singled out from each proneural cluster. Next, the paired photoreceptors R2/R5 are recruited by R8, followed by the recruitment of the R3/R4 pair and the R1/R6 pair. R7 is the last photoreceptor that is recruited into each developing ommatidium. After the recruitment of R cells, four cone cells are added to each ommatidium, and finally pigment cells are recruited (Wolff and Ready, 1993). *D-Pax2* plays an important role in the development of cone and primary pigment cells. In the *D-Pax2* mutant spa^{pol}, in which the eye-specific *spa* enhancer is deleted, development of cone and primary pigment cells is disturbed and produces a strong rough eye phenotype, the *sparkling* phenotype (Fu and Noll, 1997). The cone cell-specific transcription of *D-Pax2* is regulated by the combinatorial action of the transcription factor Lozenge and the nuclear effectors of the EGFR and Notch signaling pathways (Flores et al., 2000). *D-Pax2* and *tramtrack* control proper cone cell differentiation (Shi, 2001).

Transcriptional regulation and enhancer-promoter specificity

Development of a multi-cellular organism depends on the precise and coordinated expression of several hundreds and thousands of genes in a spatially and temporally controlled manner. This is largely regulated at the transcription level. Transcriptional activation is an intriguing and complicated process that depends on a number of *cis*-regulatory elements, which include enhancers, promoters, insulators, polycomb response elements (PREs), and trithorax response elements (TREs) (reviewed by Dorsett, 1999; Arnosti, 2003; Bondarenko et al., 2003; Schwartz and Pirrotta, 2007; Schuettengruber et al., 2007). A promoter is the minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription by the RNA polymerase II machinery in the presence of an enhancer. Typically, a promoter comprises a transcription initiation site and extends tens or hundreds of nucleotides upstream or downstream of the initiation site. There are several sequence motifs commonly found in promoters, which include the TATA box, initiator (Inr), downstream core promoter element (DPE) and TFIIB recognition element (BRE) (Juven-Gershon et al., 2008). These promoter elements are found in many but not all promoters, which suggests that other promoter elements might exist mediating transcription initiation. Apart from controlling transcription initiation, promoters can mediate the enhancer-specificity (Butler and Kadonaga, 2001).

Transcriptional enhancers are *cis*-regulatory elements responsible for gene activation specific in space and time. Often enhancers are found either upstream or downstream of promoters and are able to work in both orientations over long distances (reviewed by Serfling et al., 1985; Blackwood and Kadonaga, 1998; Dillon and Sabbattini, 2000). These elements contain binding sites for different transcriptional activators that, upon binding to these sequences, specifically activate transcription from the linked cognate promoter. In general, transcriptional activators contain a DNA binding domain that enable it to bind to a specific enhancer sequence and an activation domain that permits activation of transcription at the promoter. Activation domains can turn on transcription by either recruiting the polymerase II machinery (Ptashne and Gann, 1997) or by recruiting chromatin modifying factors that lead to an increased accessibility of the transcription initiation site to the transcription machinery (reviewed by Workman and Kingston, 1998).

An important question concerning the transcriptional regulation of a gene is how its enhancers find and specifically activate their cognate promoters rather than other non-cognate promoters (Li and Noll, 1994). Previous studies mainly suggested three models of how this

is achieved: (i) the enhancer-promoter compatibility model, (ii) the promoter competition model, and (iii) the insulator model (Fig. 2). In the first model, transcriptional activators binding to an enhancer can only interact with proteins bound to their cognate promoter but not those bound to other neighboring promoters (Fig. 2A; Li and Noll, 1994; Merli et al., 1996). In the second model, an enhancer that is capable of activating several promoters preferentially activates the strongest promoter (Fig. 2B; Calhoun et al., 2002; Calhoun and Levine, 2003; Akbari et al., 2008). The strength of a promoter depends on the specific promoter elements, like TATA box and initiator element, or on promoter-tethering elements that provide assistance in the interaction of the promoter with the enhancer. In contrast to the first model, here mutations affecting the strong promoter can lead to the activation of (the) other promoter(s). In the third model, insulator DNA elements, located between an enhancer and promoters, prevent the activation of the promoters by the enhancer (Fig. 2C; reviewed by Geyer, 1997; West et al., 2002; Gaszner and Felsenfeld, 2006). Accordingly, insulators are frequently found between adjacent genes.

Transvection

Pairing of homologous chromosomes or chromosomal regions exert profound effects on gene activation or repression, collectively termed trans-sensing effects (Tartof and Henikoff, 1991; Henikoff and Comai, 1998). In *Drosophila*, like other *Diptera*, homologous chromosomes are always paired in somatic cells (Stevens, 1908; Metz, 1916). Gene activation in *trans* is generally called transvection (reviewed by Pirrotta, 1999; Wu and Morris, 1999; Duncan, 2002; Kennison and Southworth, 2002; Sipos and Gyurkovics, 2005). In 1954, Lewis first coined the term transvection for the phenomenon of pairing-dependent inter-allelic complementation observed at the *Ultrabithorax* (*Ubx*) locus of *Drosophila* (Lewis, 1954). Since then, this phenomenon has been reported for several other genes in *Drosophila*, which include *Abdominal-B* (*Abd-B*), *apterous* (*ap*), *decapentaplegic* (*dpp*), *eyes absent* (*eya*), *Sex combs reduced* (*Scr*), *vestigial* (*vg*), *yellow* (*y*), *white*^{speckled} (w^{sp}), and *wings-up A* (*wup A*) (Korge, 1981; Gelbart, 1982; Davison et al., 1985; Geyer, 1990; Martinez-Laborda et al., 1992; Leiserson et al., 1994; Sipos et al., 1998; Morris et al., 1999; Southworth and Kennison, 2002; Coulthard et al., 2005; Gohl et al., 2008). Apart from *Drosophila*, transvection or transvection-like effects have been reported in fungi, plants and mammals, where there is no extensive homologous chromosome pairing (reviewed by Pirrotta, 1999; Wu and Morris, 1999; Duncan, 2002; Kennison and Southworth, 2002; Sipos

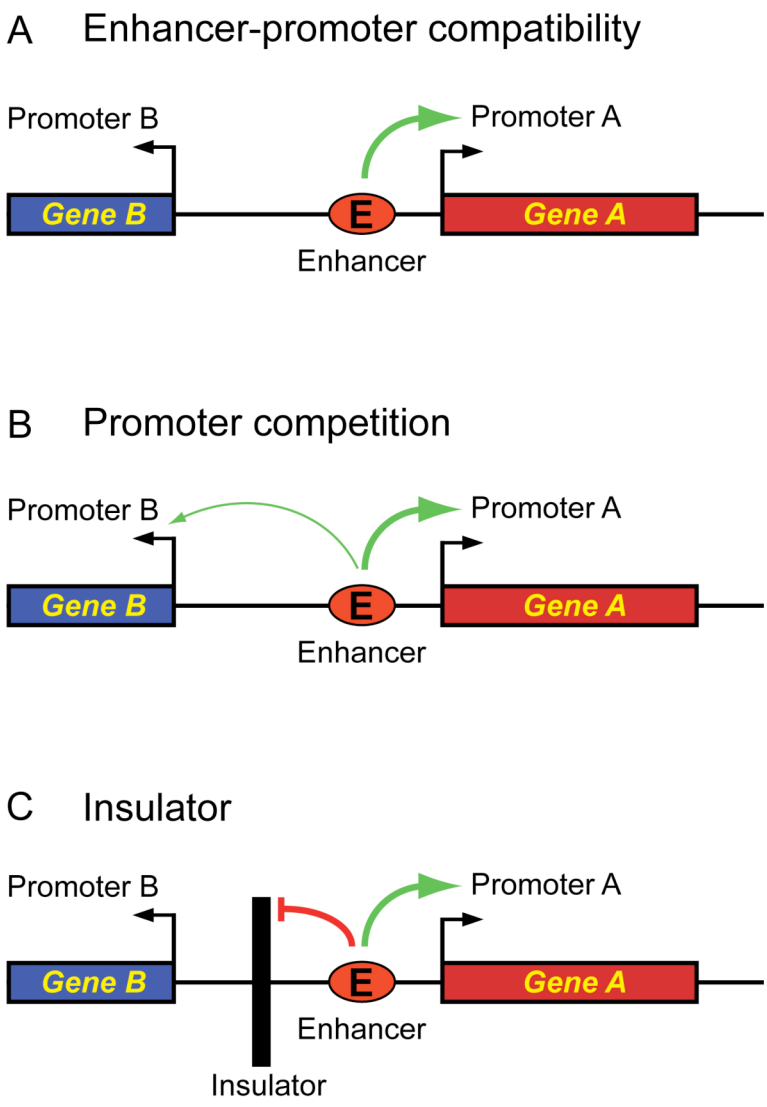

Fig. 2. **Regulation of enhancer-promoter specificity.**

Three models illustrate how the enhancer E of *gene A* can specifically target its cognate promoter A. **(A)** Specificity is achieved by the enhancer-promoter compatibility, where factors binding to the enhancer can only interact functionally with the factors binding to the cognate promoter, but not with those bound to the other promoter B. **(B)** Specificity is provided by promoter competition, where a strong promoter A competes with the weak promoter B for activation by the enhancer. **(C)** An insulator regulates the enhancer-promoter specificity. The enhancer E activates promoter A but cannot activate the promoter B because an insulator is located between the enhancer and promoter B.

and Gyurkovics, 2005). Moreover, it has been demonstrated that the entire *Drosophila* genome is generally permissive for transvection (Chen et al., 2002).

Generally, inter-allelic complementation is observed between two classes of alleles, (i) mutations of the enhancer, and (ii) mutations of the promoter or coding region (Fig. 3). Transvection studies of the *y* gene suggested two models for the mechanism of transvection (Morris et al., 1998; Morris et al., 1999). The first and widely accepted model is based on the ability of the enhancer to activate transcription from the cognate promoter on the paired homologous chromosome (Fig. 3). In the second model, an insulator inserted between the enhancer and promoter is bypassed through a change in gene structure that depends on allelic pairing (Morris et al., 1998; Morris et al., 1999).

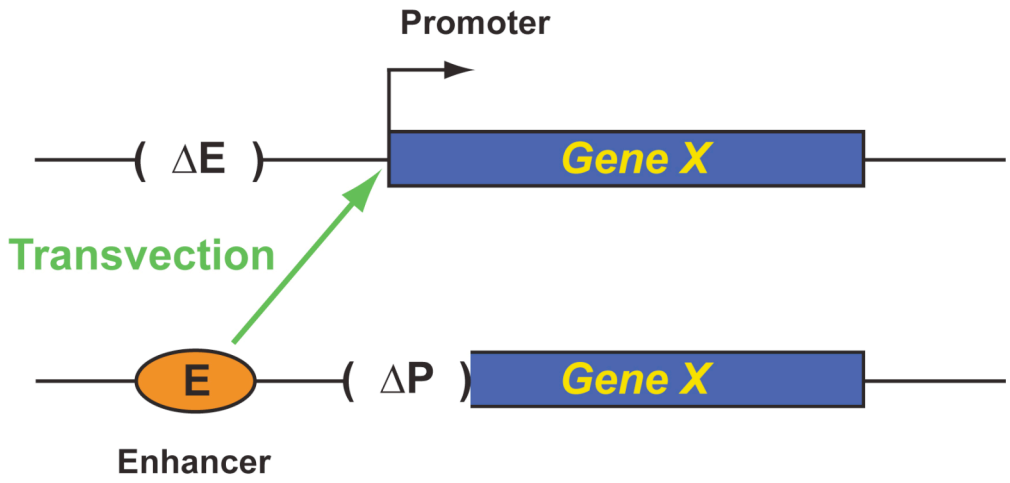

Fig. 3. Model of transvection.

The enhancer deletion allele, *ΔE*, of gene *X* is transcriptionally activated through its intact promoter by the enhancer of the paired promoter deletion allele, *ΔP*, of gene *X*. Homozygous *ΔE* or *ΔP* mutants have no gene *X* function because they lack the enhancer or promoter. However, in the transheterozygous combination of *ΔE*/*ΔP*, a functional gene *X* product is produced. This phenomenon of inter-allelic complementation, termed transvection, results from the ability of an enhancer to activate transcription from its cognate promoter located on the homologous chromosome in a pairing-dependent manner.

An important aspect of transvection is its dependence on chromosomal pairing. Disruption of allelic pairing leads to the loss of transvection. If one allele is displaced to a different chromosomal location by translocation, inter-allelic complementation is abrogated. Hence, transvection depends on close apposition of the enhancer and promoter. Apart from chromosomal pairing, most but not all the instances of transvection are dependent on the function of *zeste* (*z*) (Lewis, 1954; Gelbart and Wu, 1982; Geyer et al., 1990; Leiserson et al., 1994; Hopmann et al., 1995; Duncan, 2002; Southworth and Kennison, 2002; Coulthard et al., 2005). Zeste is a DNA binding protein that is capable of forming self-aggregates and believed to help bringing the homologous chromosomes into close proximity.

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Chapter 2

Enhancer-promoter compatibility regulates the transcriptional specificity of the *D-Pax2* gene during *Drosophila* development

Summary

Enhancer-promoter specificity has been a fundamental problem in eukaryotic gene transcription. How an enhancer specifically activates transcription from its cognate promoter is not well understood. Here, we have investigated the basis of the specificity of enhancer-promoter interactions at the *D-Pax2* locus in its own chromatin environment. The *D-Pax2* gene is transcribed from two different promoters, the PNS promoter and the CNS promoter, in a tissue-specific fashion. Interestingly, the *D-Pax2* enhancers show specificity for only one of the two promoters. The *shaven* (*sv*) enhancer, a PNS-specific enhancer, and the *sparkling* (*spa*) enhancer, an eye-specific enhancer, specifically activate the PNS-promoter and do not activate the CNS-promoter. The CNS enhancer is specific for the CNS promoter and does not activate the PNS promoter. We have also shown that the compatibility between enhancer and promoter, rather than promoter competition or insulator DNA, mediates the enhancer-promoter specificity. Finally, our results show that the promoters rather than the enhancers control the enhancer-promoter specificity.

Introduction

Development of multicellular organisms requires precise and coordinated activation of thousands of genes in a spatially and temporally regulated manner. This is controlled by a number of *cis*-regulatory elements, including enhancers, promoters, insulators, polycomb response elements (PREs), and trithorax response elements (TREs) (Reviewed by Dorsett, 1999; Arnosti, 2003; Bondarenko et al., 2003; Schwartz and Pirrotta, 2007; Schuettengruber et al., 2007). Transcriptional enhancers are *cis*-regulatory DNA elements responsible for the spatial and temporal specific activation of gene expression. Enhancers are often found upstream or downstream of the transcription initiation site and able to work in both orientations at long-range (reviewed by Serfling et al., 1985; Ptashne, 1986; Blackwood and Kadonaga, 1998; Sipos and Gyurkovics, 2005; Dillon and Sabbattini, 2000). Enhancers contain cluster of binding sites for specific transcription factors, which upon binding to these sequences recruit other activators or chromatin modifiers leading to the initiation of transcription by RNA polymerase II (RNAP II) from the linked core promoter. A core promoter contains distinct core promoter sequence elements that mediate recruitment of the general transcription machinery and specify the accurate RNAP II transcription initiation (reviewed by Butler and Kadonaga, 2002). Several core promoter elements have been discovered, which include the TATA box, the initiator element, and the downstream promoter element (DPE) (Kadonaga, 2002).

One of the intriguing properties of enhancers is that they specifically activate their cognate promoter rather than other promoters, for example, that of a neighboring gene. Studies on enhancer trap lines and position effects of transgenes suggest that several enhancers can also activate other heterologous promoters (Kania et al., 1995; Bier et al., 1989; Lee and Wu, 2006). DNA aberrations, like gene displacements or mutations in *cis*-regulatory elements, disturb the normal gene expression that may lead to a diseased state (reviewed by Kleinjan and van Heyningen, 2005). Hence, studies elucidating the mechanism of transcriptional regulation are indispensable. Despite considerable progress in understanding the transcriptional regulation of genes, the mechanism controlling enhancer-promoter specificity is not well understood.

Previous work has proposed mainly three different models for the enhancer-promoter specificity: (i) the enhancer-promoter compatibility, (ii) promoter competition, and (iii)

insulator DNA elements. In the first model, the individual properties of enhancers and promoters determine the transcriptional specificity (Li and Noll, 1994; Merli et al., 1996). In other words, the proteins binding to the enhancer form a stable and functional interaction only with the proteins binding at the cognate promoter, but not with the proteins binding at other promoters. In the second model, a shared enhancer capable of activating several promoters prefers to activate only one promoter, which is then called the strongest promoter (Calhoun et al., 2002; Calhoun and Levine, 2003). Here the various promoters compete for interaction with the same enhancer and the strongest interaction wins. If one removes the strongest promoter, one of the other promoters will be activated, whereas in the enhancer-promoter compatibility model removal of the activated promoter does not result in the activation of one of the others. Promoter proximal tethering elements have been discovered that regulate this type of promoter-preference (Calhoun et al., 2002; Lin et al., 2003; Akbari et al. 2008). In the third model, insulator DNA elements block nonspecific enhancer-promoter interactions. Insulators are DNA elements, which block the activation of a gene by an enhancer when they are located between the enhancer and the promoter of the gene (reviewed by Geyer, 1997; West et al., 2002; Gaszner and Felsenfeld, 2006). Generally, insulators are found between genes, such that enhancers of a particular gene are allowed to activate only their cognate promoter.

In this study, we have analyzed the enhancer-promoter specificity at a single gene locus, that of the *D-Pax2* gene. Interestingly, two different tissue-specific enhancers of *D-Pax2* activate only one of the two promoters of the gene. We have found that the transcriptional specificity at this locus is established by the compatibility between the enhancers and promoters rather than by promoter competition or through an insulator DNA element. Finally, we have shown that it is the promoters rather than the enhancers that regulate the enhancer-promoter specificity.

Results

The *cis*-regulatory elements of the *D-Pax2* locus

The *D-Pax2* gene, also known as *shaven* (*sv*) or *sparkling* (*spa*), is the *Drosophila* homolog of the vertebrate *Pax-2/5/8* subfamily. Thus, it encodes a paired-domain containing

transcription factor (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999). Like its mammalian counterpart, *D-Pax2* plays an important role in several developing sensory organs (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999). In *Drosophila*, *D-Pax2* is expressed in the developing embryonic and larval peripheral nervous system (PNS) as well as in the corresponding larval and adult sensory organs. In adult mechanosensory bristles, it regulates the proper specification and differentiation of the shaft and sheath cells (Fu et al., 1998; Kavaler et al., 1999). The *sv* enhancer regulating *D-Pax2* expression in the PNS has been mapped upstream of the PNS promoter (Fig. 1; Kavaler et al., 1999; Shi, 2001). The two hypomorphic *sv* alleles, *shaven-naked* (sv^{n}) and *shaven-depilate* (sv^{de}), which contain insertions of transposable elements in the *sv* enhancer, display the *shaven* phenotype, i.e., strong reduction and loss of bristles all over the body (Fu et al., 1998). Apart from its role in the PNS, *D-Pax2* also plays an important role in eye development, where its function is required for the proper specification and differentiation of the cone and primary pigment cells (Fu and Noll, 1997, Flores et al., 2000). The eye-specific *spa* enhancer has been mapped to the 4th-intron (Fig. 1; Fu and Noll, 1997). The spa^{pol} allele, which is a deletion uncovering the third and fourth exon and the *spa* enhancer (Fig. 1), displays a strong rough eye phenotype (Fu and Noll, 1997). Previously the *Drosophila sv* and *spa* alleles were thought to represent two different genes, but our lab has shown that they are mutations in two different tissue-specific enhancers of the *D-Pax2* gene (Fu et al., 1998). In addition to its expression in the PNS and eye, *D-Pax2* is expressed in the embryonic CNS (Fu and Noll, 1997).

D-Pax2 is transcribed from two different promoters

Previously it was thought that *D-Pax2* is transcribed from a single promoter. However, studies based on two *D-Pax2* point mutant alleles, sv^{E67} and sv^{E69}, and a promoter deletion allele, $sv^{\Delta 122}$, led to the discovery of another promoter of the *D-Pax2* locus. The sv^{E67} allele contains a premature stop codon in the 4th exon, while the sv^{E69} allele contains a stop codon in the 7th exon. Homozygous sv^{E69} embryos showed complete loss of D-Pax2 expression in PNS and CNS, whereas in sv^{E67} embryos expression is abolished only in the PNS, but not in CNS. This suggested that there is another promoter from which *D-Pax2* is transcribed in the CNS (data not shown, E. Frei, M. Daube, and M. Noll, unpublished). The $sv^{\Delta 122}$ allele was generated by E. Frei and M. Daube by imprecise excision of the P-element *l(4)2C2* (kindly provided by J. Kronhamn and A. Rasmuson-Lestander), located 1 kb upstream of the *D-Pax2* transcription start site (Fig. 1). Molecular characterization of this allele detected a deletion of

10 kb uncovering the *D-Pax2* promoter and first four exons including the *spa* enhancer, whereas the entire P-element remains intact and inserted at the same site as in *l(4)2C2* (Fig. 1). Immunostaining of $sv^{\Delta 122}$ embryos with anti-D-Pax2 antiserum revealed that D-Pax2 expression in the PNS is completely abolished, whereas its expression in the CNS is not affected (Fig. 3A,B). This confirms that the *D-Pax2* gene is transcribed in the CNS from a promoter outside the region deleted in the $sv^{\Delta 122}$ allele. As the *sv* enhancer probably remains functional in $sv^{\Delta 122}$ embryos (Shi, 2001), this result further suggests that transcription in the PNS depends on the other promoter, the PNS promoter. Subsequent molecular characterization of *D-Pax2* transcripts by 5' RACE confirmed the prediction of another first exon in intron 4 whose transcriptional initiation site is located 9729 bp downstream of the other transcriptional start site (E. Frei, M. Daube, and M. Noll, unpublished). This new exon is spliced to the old exon 5 of the common downstream region (Fig. 1).

Enhancer-promoter specificity at the *D-Pax2* locus

These results confirm that the *sv* enhancer activates *D-Pax2* transcription from the PNS promoter (Fu et al., 1998; Kavaler et al., 1999). To test whether the *sv* enhancer is able to activate transcription from the other promoter, we performed whole mount *in situ* hybridization to *Drosophila* embryos with specific probes that cover exons 2-4 (P1), the newly discovered first exon transcribed in the CNS (P2), and common downstream exons (P3) (Fig. 1). Probe P1 detected a signal exclusively in the PNS, but none in the CNS (Fig. 2A), which shows that the probe is specific for PNS transcripts. This result further excludes that the CNS enhancer also activates transcription from the PNS promoter and thus implies that transcription in the CNS depends on the other promoter. By contrast probe P2 produced a signal only in the CNS (Fig. 2B), which implies that neither the *sv* nor the *spa* enhancer is able to activate transcription also from the CNS promoter. As expected, the probe P3, covering common exons, detected signals in the PNS and CNS (Fig. 2C) in a pattern similar to the wild-type D-Pax2 expression pattern detected by immunostaining (Fig. 2D). In summary, these results strongly suggest a specificity of the interaction between enhancers and their cognate promoters at the *D-Pax2* locus. The *sv* enhancer specifically activates transcription only from the PNS promoter while the CNS enhancer activates only the CNS promoter.

The *sv* enhancer is specific for the PNS promoter

To further validate the *sv* enhancer specificity for the PNS promoter, a PNS promoter deletion allele, sv^{11A}, was generated by imprecise excision of the P-element *l(4)2C2* (Fig. 1). The molecular characterization of the sv^{11A} allele shows that it contains a deletion of 2.2 kb from –1064 bp to +1194 bp, uncovering the PNS promoter and first exon (Fig. 1). Similar to $sv^{\Delta 122}$ homozygotes, homozygous sv^{11A} animals die as first-instar larvae. All external sensory organs of these larvae appear to be degenerated (data not shown). Homozygous sv^{11A} embryos do not exhibit any detectable D-Pax2 expression in the PNS, whereas its expression in the CNS is unaffected (Fig. 3C). Hence, the CNS transcription unit is intact in the sv^{11A} allele (Fig. 1). In summary, these results demonstrate (i) that the *sv* enhancer is specific for the PNS promoter, and (ii) that this *sv* enhancer-PNS promoter specificity is not regulated by promoter competition, as the *sv* enhancer does not activate the CNS promoter even in the absence of the PNS promoter.

We further confirmed the specificity of the *sv* enhancer for the PNS promoter by RT-PCR analysis of *D-Pax2* transcripts in pupal wing discs where *D-Pax2* is expressed in the developing mechanosensory bristles (Fu et al., 1998; Kavaler et al., 1999). RT-PCR with CNS-specific primers of total RNA isolated from *y w* early pupal wing discs did not produce any product, whereas the product of 380 bp derived from the CNS transcript was observed when total RNA from *y w* embryos was used (Fig. 3E). By contrast, RT-PCR of total RNA from sv^{11A} embryos, in which CNS expression remains unaffected (Fig. 3C), produced the expected CNS-specific product (Fig. 3E). Thus, the absence of CNS-specific transcripts in wing discs confirms that the *sv* enhancer is specific for the PNS promoter and does not activate the CNS promoter.

The *sv* enhancer does not activate the CNS promoter even in close proximity

Generally enhancers are capable of activating their cognate promoters even if separated by large distances. However, for some enhancers the distance from the promoter is crucial for their activation strength (Dillon et al., 1997; Kmita et al., 2002). Hence, we asked whether the distance of 10 kb between the *sv* enhancer and CNS promoter in the wild type might prevent activation of the CNS promoter by the *sv* enhancer. To answer this question, we analyzed D-Pax2 expression in $sv^{\Delta 122\text{-}P}$ embryos. In the $sv^{\Delta 122\text{-}P}$ allele, the *sv* enhancer and the CNS promoter were brought to a distance of 1 kb by precise excision of the P-element in the

$sv^{\Delta 122}$ allele (Fig. 1). Thus, the $sv^{\Delta 122\text{-}P}$ allele contains a 10 kb deletion identical to that of the $sv^{\Delta 122}$ allele, except that the P-element is excised. In homozygous $sv^{\Delta 122\text{-}P}$ embryos, D-Pax2 expression is not detectable in the PNS, whereas its expression in the CNS remains unaffected (Fig. 3D). This result suggests that the *sv* enhancer does not activate transcription from the CNS promoter, even when they are in close proximity. We conclude that the *sv* enhancer is specific for the PNS promoter and does not activate the CNS promoter. In addition, the promoter competition mechanism can be excluded in this case to be responsible for the enhancer-promoter specificity.

The *spa* enhancer is specific for the PNS promoter

Based on our previous work, we knew that the *spa* enhancer activates transcription from the PNS promoter. The rescue construct, in which D-Pax2 is expressed under the control of the *spa* enhancer and PNS promoter, completely rescues the eye phenotype of spa^{pol} flies (Fu and Noll, 1997). We next asked whether the *spa* enhancer is able to activate D-Pax2 expression from the CNS promoter. In order to answer this question, we took advantage of the PNS promoter deletion allele, sv^{11A}. We reasoned if the *spa* enhancer is able to activate transcription from the CNS promoter, the eye phenotype of sv^{11A} animals should be wild-type.

However, homozygous sv^{11A} animals die as first instar larvae. Therefore, these animals were rescued to fertile adults by providing the *D-Pax2* PNS function through the *6.7-spa* transgene, which expresses D-Pax2 under the control of a 6.7 kb upstream region of *D-Pax2*. This transgene completely rescues the PNS function but not the eye-specific function of *D-Pax2* (Fu et al., 1998). These rescued *6.7-spa*/+; sv^{11A} flies showed rough eyes similar to the *spa* enhancer deletion allele spa^{pol}, although the eye phenotype appeared to be slightly rescued (Fig. 4A-C). This is explained by a low level of expression of *D-Pax2* from the *6.7-spa* transgene in the developing eye (Shi, 2001). Consistent with this explanation, even spa^{pol} eyes were similarly rescued by the *6.7-spa* transgene (Fig. 4D). Therefore, these results suggest that the *spa* enhancer does not activate the CNS promoter to rescue the eye phenotype, even though the *spa* enhancer is close to the CNS promoter.

It could be argued that the D-Pax2 protein translated from the CNS transcripts is not able to rescue the eye phenotype. However, we consider this possibility very improbable because

the spa^{pol} allele, which is an in-frame deletion of the coding exons 3 and 4, produces *D-Pax2* transcripts that are functional in the PNS, as evident from spa^{pol} flies, and functional in the eye, as evident from sv^{11A}/spa^{pol} flies (see Chapter 3). This suggests that the N-terminal part of the D-Pax2 protein is not necessary for the PNS and eye functions and hence that the *spa* enhancer cannot activate the CNS promoter. In addition, we tested the *spa* enhancer specificity for the PNS promoter through a method different from rescue analysis. If the *spa* enhancer activates transcription from the CNS promoter, CNS-specific transcripts should be detectable in eye discs. To determine this, we performed RT-PCR with CNS specific primers and total RNA isolated from eye discs of *y w*, spa^{pol}, and sv^{11A}/spa^{pol} third instar larvae. As expected, none of these third instar eye disc RNAs produced any products by RT-PCR, whereas total RNA isolated from *y w* embryos generated a product of the expected size (Fig. 4E). This result suggests that the CNS-specific transcripts are not produced in eye imaginal discs, which confirms that the *spa* enhancer does not activate transcription from the CNS promoter. In summary, we conclude that the *sv* enhancer and the *spa* enhancer activate transcription specifically through the PNS promoter but not the CNS promoter.

Mechanism governing the enhancer-promoter specificity at the *D-Pax2* locus

Thus, neither the *sv* enhancer nor the *spa* enhancer are able to activate the CNS promoter. This is true even in the absence of the PNS promoter, which suggest that there is no competition between these promoters and hence excludes that the promoter competition mechanism regulates the enhancer-promoter specificity at the *D-Pax2* locus. Therefore, the enhancer-promoter specificity is achieved by one of the remaining two mechanisms, illustrated by the enhancer-promoter compatibility or insulator DNA model (Fig. 5). If an insulator DNA is regulating the transcriptional specificity at the *D-Pax2* locus, it would serve its purpose only in a position upstream of the CNS promoter since it would have to prevent the upstream *sv* and *spa* enhancers from activating the CNS promoter. To test whether an insulator DNA element is present upstream of the CNS promoter, we used the *PlacW* enhancer trap line, *l(4)2C2*, that expresses *lacZ* through a P-element promoter (Bier et al., 1989). The LacZ expression pattern of *l(4)2C2* embryos, visualized by anti-lacZ immunostaining, revealed that it resembles the corresponding D-Pax2 expression pattern in the embryonic PNS and CNS (Fig. 6). In the PNS, most of the LacZ expressing cells co-localized with D-Pax2 expressing cells (Fig. 6A-D). This demonstrates that the *sv* enhancer can faithfully activate the nearby P-element promoter in nearly all cells expressing D-Pax2 in the PNS. Similarly, the CNS enhancer can drive LacZ expression through the P-element

promoter in the embryonic CNS (Fig. 6E-G). Many but not all LacZ-expressing cells co-localized with D-Pax2-expressing cells, which suggests that the CNS enhancer can also properly activate the P-element promoter, though not in all cells. We therefore consider the P-element promoter to be 'promiscuous'. We have further analyzed the co-localization of D-Pax2 and lacZ by ImageJ software, which confirmed the co-localization, depicted as white pixels in the PNS and the CNS (Fig. 6D,H). Thus, the CNS enhancer is able to activate the P-element promoter, which is located at a distance of more than 20 kb, a region that moreover includes several promoters, namely the PNS promoter, the *white* gene promoter, and the CNS promoter (Fig. 1). This is evident from the fact, that the CNS enhancer is outside this region deleted by the $sv^{\Delta 122}$ allele (s. above). Thus, these results suggest that both the *sv* enhancer and the CNS enhancer can drive the P-element promoter in a tissue-specific fashion. This observation, however, cannot be explained by the mere presence of an insulator upstream of the CNS promoter because of the following arguments. If the CNS enhancer is located downstream of the CNS promoter, it could not have activated the P-element promoter in the presence of this insulator. Even if the CNS enhancer is located upstream of the CNS promoter, the presence of insulator cannot explain all results because the CNS enhancer would have to act across this insulator to activate its cognate promoter. A normal insulator prevents all enhancers from activating a promoter when located between the enhancer and the promoter (Geyer, 1997; West et al., 2002). Therefore, we exclude the possibility of a normal insulator element regulating the enhancer-promoter specificity. In conclusion, these results suggest that it is the compatibility between the enhancers and promoters that determines the enhancer-promoter specificity at the *D-Pax2* locus.

Promoters rather than enhancers control the enhancer-promoter specificity

These results clearly demonstrate that the *sv* enhancer is specific for the PNS promoter and the CNS enhancer is specific to the CNS promoter. Even though these enhancers show specificity for their cognate promoters, interestingly, both enhancers activate a promiscuous P-element promoter driving *lacZ* in *l(4)2C2*. LacZ expression from *l(4)2C2* resembled D-Pax2 expression in the PNS and CNS. Therefore, the promoters rather than the enhancers regulate the enhancer-promoter specificity at the *D-Pax2* locus. The enhancers regulate the tissue-specific transcriptional activation, while the promoters control the enhancer-specificity of transcriptional initiation.

Discussion

In the present study, we found enhancer-promoter specificity within a single gene locus, *D-Pax2*, regulating its tissue-specific expression pattern. The *D-Pax2* gene is transcribed from two different promoters, the PNS promoter and the CNS promoter, in a tissue-specific fashion. Interestingly, the *sv* enhancer and the *spa* enhancer activate only the PNS-promoter and do not activate the CNS-promoter. Even though the *spa* enhancer is close to the CNS promoter, it activates only the PNS promoter. Even the CNS enhancer is specific for its cognate CNS promoter and does not activate the PNS promoter, as the shorter *D-Pax2* transcripts were detected only in the CNS. We have further studied the mechanisms regulating this enhancer-promoter specificity at the *D-Pax2* locus.

How is the enhancer-promoter specificity achieved has been a fundamental question in the study of the transcriptional control of genes. Previous work has proposed three different mechanisms for the regulation of the enhancer-promoter specificity. Investigating the mechanism mediating the enhancer-promoter specificity at the *D-Pax2* locus, we have excluded the promoter competition mechanism since deletion of the PNS promoter does not change the specificity of the *sv* or *spa* enhancer in favor of the CNS promoter. Using the *PlacW* enhancer-trap insertion, we have further ruled out the possibility that a general insulator DNA element regulates the enhancer-promoter specificity because the CNS enhancer can activate the *lacZ* gene located upstream of the PNS promoter. This observation cannot be explained by the presence of a normal insulator DNA element that would have to be located between them to ensure specificity of the CNS enhancer for its cognate promoter. However, we cannot rule out the possibility of a special one-way insulator that would block activation by an enhancer in only one direction but not the other. Such an insulator might prevent the upstream *sv* and *spa* enhancers from activating the CNS promoter but allow the downstream CNS enhancer to activate the upstream P-element promoter. An insulator with this property has not been identified in *Drosophila*. In mice, however, such an insulator element, called polar silencer, has been reported to be implicated in the transcriptional regulation of the *HoxD* complex (Kmita et al., 2000). Nevertheless, the presence of a one-way insulator cannot explain the inability of the CNS enhancer to activate the PNS promoter. Therefore, we rule out that an insulator DNA element mediates the enhancer-promoter specificity at the *D-Pax2* locus. Taken together, our results suggest that it is the compatibility between the enhancer and promoter that mediates the transcriptional specificity at the *D-Pax2*

locus. We propose that the transcription factors bound to the *sv* enhancer can only recognize the factors bound to the PNS promoter, and similarly that the factors bound to the CNS enhancer specifically recognize those bound to the CNS promoter, as in a lock and key model.

Our results add another case to previous findings where the intrinsic properties of the enhancers and promoters regulate the enhancer-promoter specificity (Li and Noll, 1994; Merli et al., 1996). Our lab has investigated the question of the mechanism by which the promoter-enhancer specificity is achieved for the first time and found that it is the compatibility between the enhancer and its cognate promoter that ensures transcriptional specificity at the closely linked genes of the *gooseberry* (*gsb*) and *gooseberry neuro* (*gsbn*) locus of *Drosophila* (Li and Noll, 1994). It has been shown that some enhancers prefer to activate TATA box-containing promoters to initiator containing promoters and vice versa (Ohtsuki et al., 1998; Butler and Kadonaga, 2001). We speculate that there might be some tissue-specific promoter elements, which are part of these promoters, that would allow only transcriptional activation by the corresponding enhancer. Recently, a promoter-tethering element has been reported, located in the promoter region of the *Abd-B* promoter, that is capable of selectively recruiting *iab* enhancers to the cognate promoter (Akbari et al., 2008). Finally, we have demonstrated that promoters rather than the enhancers control enhancer-promoter specificity, which is in line with the previous findings that core promoter elements apart from regulating the transcription initiation also play a significant role in the enhancer-promoter specificity (Butler and Kadonaga, 2002).

In summary, our data demonstrate that the compatibility between the enhancer and promoter mediates the enhancer-promoter specificity at the *D-Pax2* locus. We provide the first evidence in *Drosophila* that enhancer-promoter specificity within a normal gene locus is controlled by the core properties of the *cis*-regulatory elements.

Materials and methods

Fly stocks

The following fly stocks were used. *y w*, *OreR*, sv^{11A}, $sv^{\Delta 122}$, $sv^{\Delta 122\text{-}P}$, spa^{pol}, *y w*; *6.7-spa* (3rd

chromosome), *y w*; *l(4)2C2/ci*^{D} *spa*^{pol}, and *w*^{1118}; *P{ActGFP}unc-13*^{GJ}*/sv*^{11A}. A GFP-marked 4th-chromosome, obtained from the Bloomington Stock Center, stock BL-9549, was used to isolate homozygous *sv*^{11A} embryos.

P-element-generated alleles

The P element *l(4)2C2* is inserted 1064 bp upstream of the *D-Pax2* gene (Fig. 2). The P-element line was crossed to *Δ2–3* to generate excisions. The male jump-starters were tested for complementation with *spa*^{pol}. The F1 excision lines, which showed a very weak rough eye phenotype at the posterior end over the *spa*^{pol}, were selected for further analysis. The molecular nature of these alleles was further analyzed by DNA sequencing of PCR products to determine the deletion breakpoints. The *sv*^{11A} allele contains a 2.2 kb deletion, from -1064 bp to +1194 bp, uncovers the entire *D-Pax2* PNS promoter and the first exon. After imprecise excision, a 20 bp sequence was inserted at the P-element insertion point.

The *sv*$^{\Delta 122}$ allele was generated by imprecise excision of the P-element in *l(4)2C2* by a similar crossing scheme. This allele did not complement the *spa*^{pol} allele. Molecular characterization of this allele showed a deletion of 10 kb region whose upstream breakpoint is located at the insertion site of the P-element. This deletion uncovers the PNS promoter, exons 1-4 along with the *spa* enhancer. This allele contains a reinsertion of the entire P-element at same location after the imprecise excision. The *sv*$^{\Delta 122\text{-}P}$ allele was generated by the precise excision of the P-element in the *sv*$^{\Delta 122}$ allele, screened for lack of the *w*$^{+}$ marker. The molecular nature of this allele was determined by PCR, which displayed complete loss of P-element and a deletion identical to that of the *sv*$^{\Delta 122}$ allele.

The generation of *sv*$^{\Delta 122\text{-}P}$ was not trivial, as described by Erich Frei who performed this experiment with Michael Daube in our lab. Surprisingly, *sv*$^{\Delta 122}$ flies have white (not even weakly red) eyes although the *w*$^{+}$ P-element *PlacW* is still present which became clear only after molecular cloning and mapping. Apparently the *w*$^{+}$ gene of *PlacW* is silenced *sv*$^{\Delta 122}$ flies, whereas it is active and visible as weak *w*$^{+}$ eyes in its parent line, *l(4)2C2*. When the *w*$^{+}$ P-element of *sv*$^{\Delta 122}$ is mobilized by *Δ2–3* P-element transposase, the flies have red-white mosaic eyes because in some eye cells the *w*$^{+}$ *PlacW* has jumped and reinserted at other loci in the genome where it is not silenced. Offspring of such flies which still carry the *Δ2–3*

transposase but have white and non-mosaic eyes were candidates for having lost the *PlacW*. The $sv^{\Delta 122\text{-}P}$ allele was identified among such candidates.

RNA *in situ* hybridization

In situ hybridization to whole mount embryos with DIG-labeled *D-Pax2* antisense RNA probe was carried out as described (Fu and Noll, 1997).

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from *Drosophila* embryos (stage 14-17), third instar larval eye discs, and wing discs of early white pupae by the use of the "Nucleospin RNA II" kit (Macherey-Nagel) according to the manufacturer's instructions. RT-PCR was performed by the use of the One Step RT-PCR kit (Qiagen), following the manufacturer's protocol. 100 ng of total RNA was used for each RT-PCR reaction. Briefly, RNA was reverse-transcribed for 30 min at 50°C, the PCR step was activated by heating for 15 min at 95°C, and the PCR was performed for 32 cycles with 1 min denaturation at 94°C, 1 min annealing at 57°C, and 1 min extension at 72°C. The PCR products were analyzed on agarose gels. The following primers were used in the RT-PCR analysis. *D-Pax2* CNS for: 5'-GTTCCGGCATCGATTACAGGT-3' (C1 in Fig. 1), *D-Pax2* CNS rev: 5'- GACGTGATATGTCACATGGGCGGAC-3' (C2 in Fig. 1). *RpL17A*, a constitutively expressed ribosomal protein gene of *Drosophila*, was used as positive control. *RpL17A* for: 5'-TGATGAACTGTGCCGACAAC-3', *RpL17A* rev: 5'-TGCATTGGATGCAATACGGG-3'.

Immunohistochemistry and microscopy

Embryos were fixed and stained as described previously (Fu and Noll, 1997). The following antibodies were used in this study. Rabbit anti-D-Pax2 antiserum (Fu and Noll, 1997), rabbit anti-LacZ polyclonal antibody (Cappel) used at a 1:2000 dilution, and chicken polyclonal anti-LacZ antibody (Abcam) used at a 1:250 dilution. Alexa 488- and Alexa 594-conjugated goat secondary antibodies (Invitrogen) were used at a 1:500 dilution. Biotinylated secondary antibodies against rabbit IgG (Vector Laboratories, Inc.) were used at a 1:300 dilution. For the color reaction, Vectastain ABC Kit (Vector Laboratories, Inc.) was used. Anti-D-Pax2 staining was enhanced by TSA^{TM} kit (Invitrogen). Microscopy was carried out with a LEICA TCS SP confocal microscope. Images were processed with Adobe Photoshop 7.0 and ImageJ software.

Scanning electron microscopy

Pictures of adult fly eyes were taken with a JEOL JSM-6360 LV scanning electron microscope. Female left eyes were pictured at a magnification of 220x.

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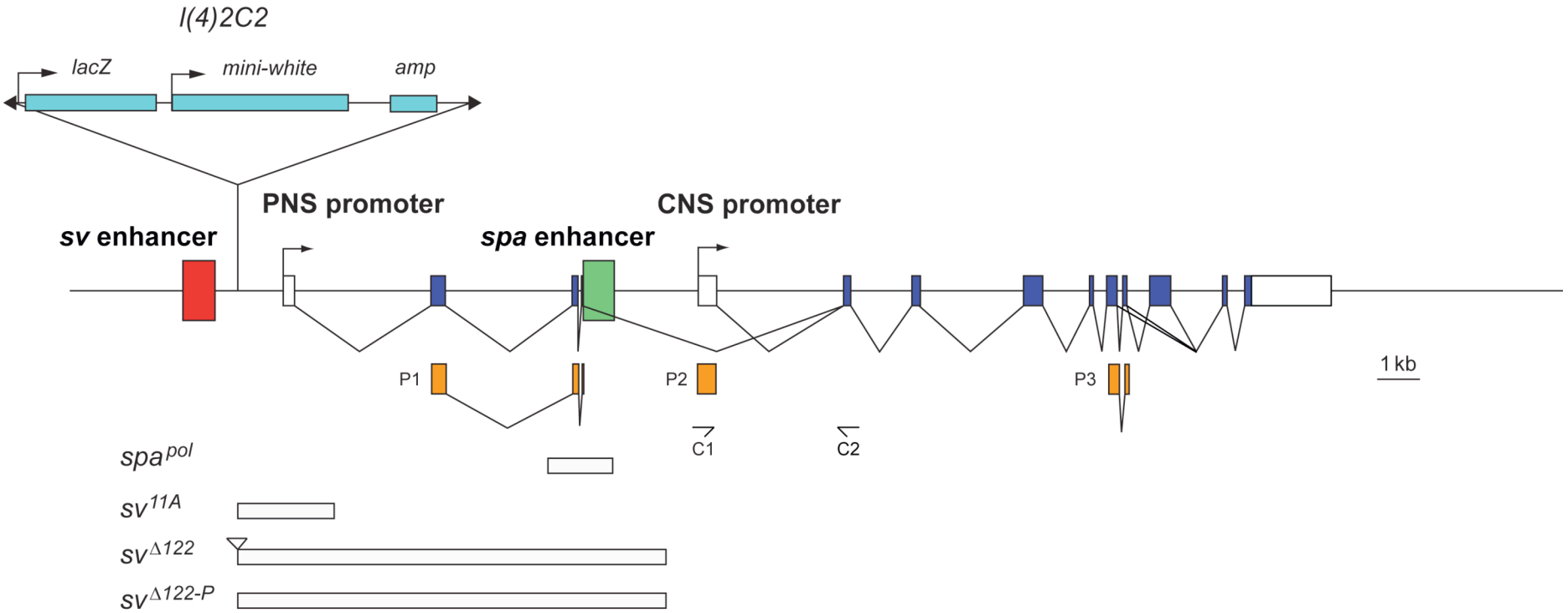

Fig. 1. Molecular and genetic map of *D-Pax2* locus.

The *D-Pax2* locus, located on the 4th chromosome of *Drosophila*, including its two promoters represented as PNS and CNS promoters. Coding regions are in blue. The open boxes below represent deletions, either the spontaneous spa^{pol} deficiency or regions deleted in *sv* mutants generated by imprecise excision of the P-element insertion *l(4)2C2* shown above the gene. The triangle at the left end of one deficiency indicates that the P-element insertion remained intact at its original location. The antisense probes P1-P3, used for the *in situ* hybridizations, are shown as orange boxes. Enhancers are depicted in red (*sv* enhancer) or green (*spa* enhancer) boxes.

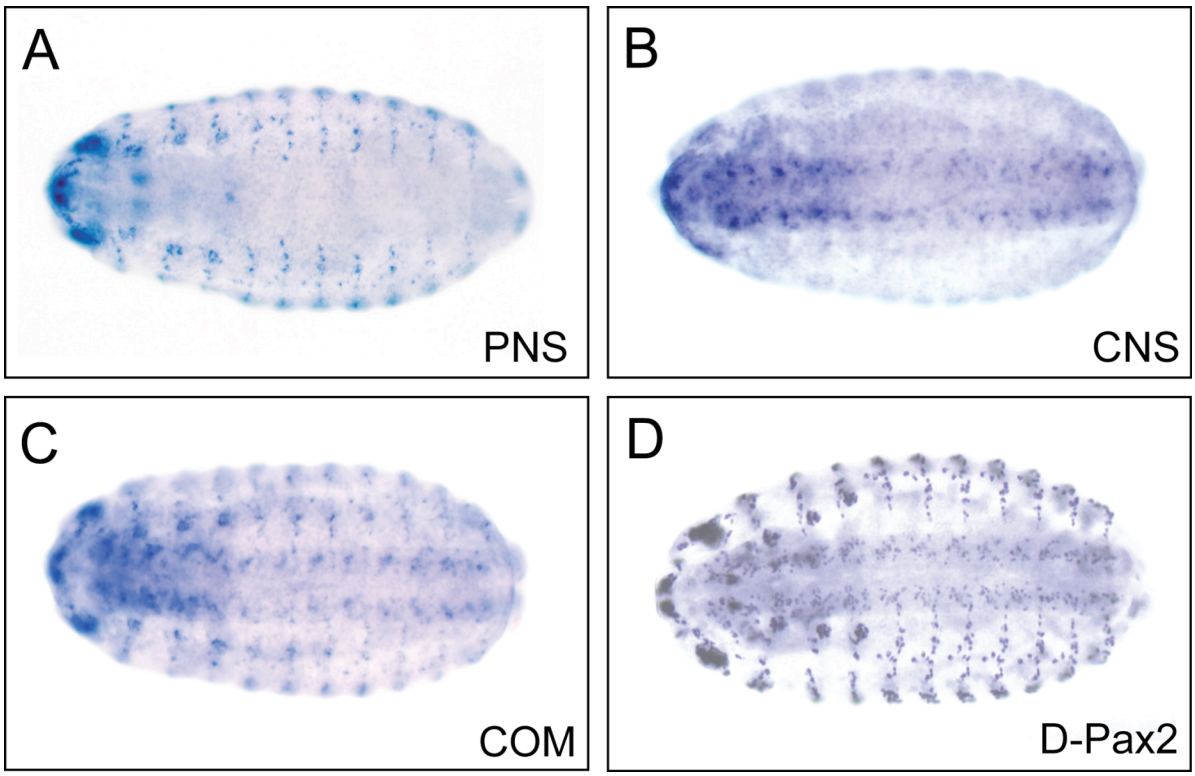

Fig. 2. Enhancer-promoter specificity at the *D-Pax2* locus.

(A-D) Whole-mount *in situ* hybridizations of wild-type embryos performed with digoxigenin-labeled *D-Pax2* antisense RNA probes. Ventral views of stage 15 embryos, oriented with their anterior to the left, are shown. The *D-Pax2* PNS-specific transcripts are detected exclusively in the PNS (A). The *D-Pax2* CNS-specific transcripts are detected only in the CNS (B). The *D-Pax2* common probe shows signals in the PNS and CNS (C). Expression of D-Pax2 determined by staining with the anti-D-Pax2 anti-serum (D).

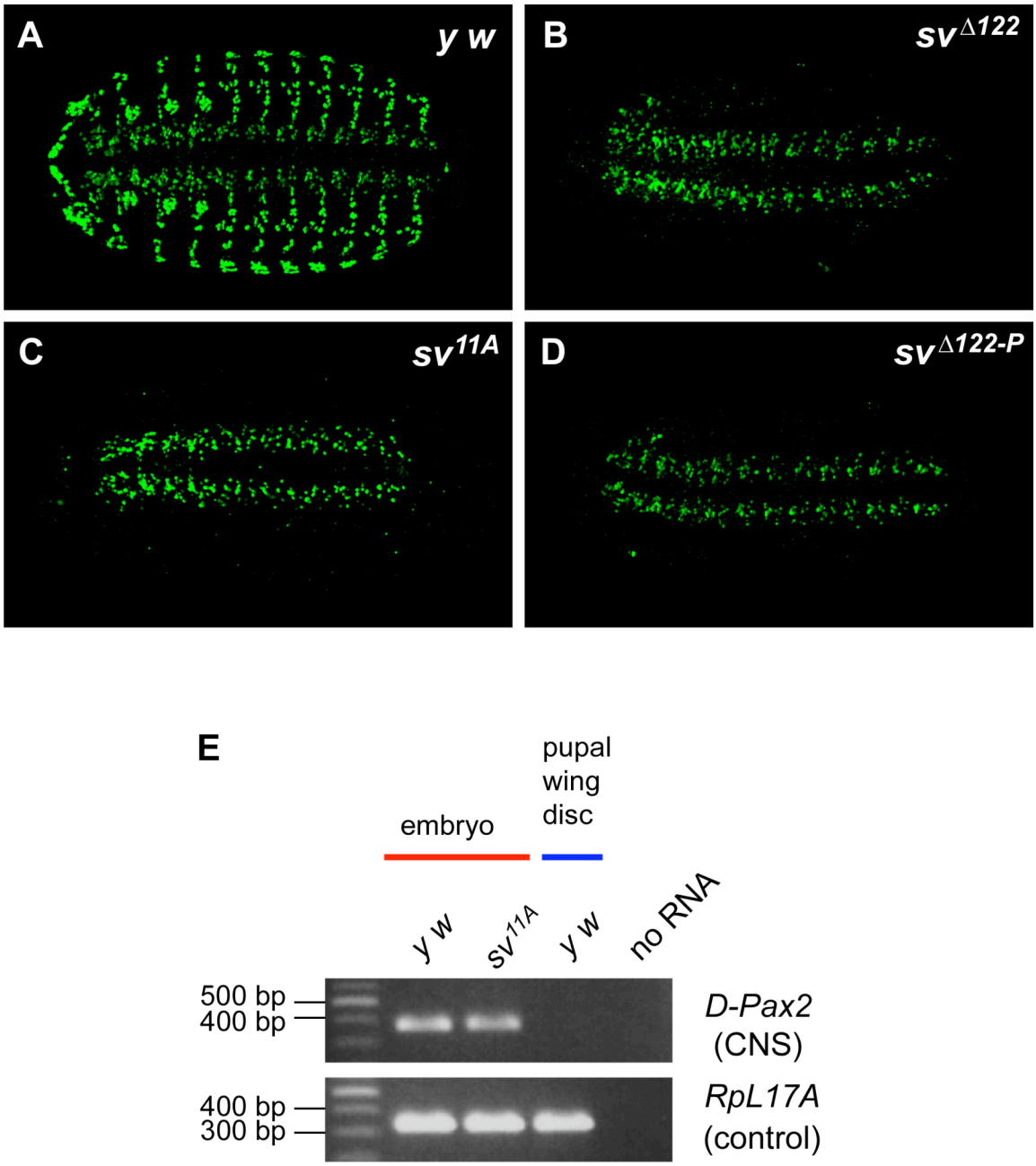

Fig. 3. The *sv* enhancer is specific for the PNS promoter of *D-Pax2*.

(A-D) Confocal images of *Drosophila* embryos (stage 16) stained with anti-D-Pax2 antiserum. In *y w* embryos, D-Pax2 expression is detectable in the PNS and CNS (A). In deletion mutant embryos with deletions removing the PNS promoter, D-Pax2 is not detectable in the PNS, whereas its expression is detectable in CNS (B-D). In all panels, ventral views are shown with anterior to the left. **(E)** RT-PCR analysis of CNS-specific *D-Pax2* transcripts in total RNA isolated from embryos and pupal wing discs.

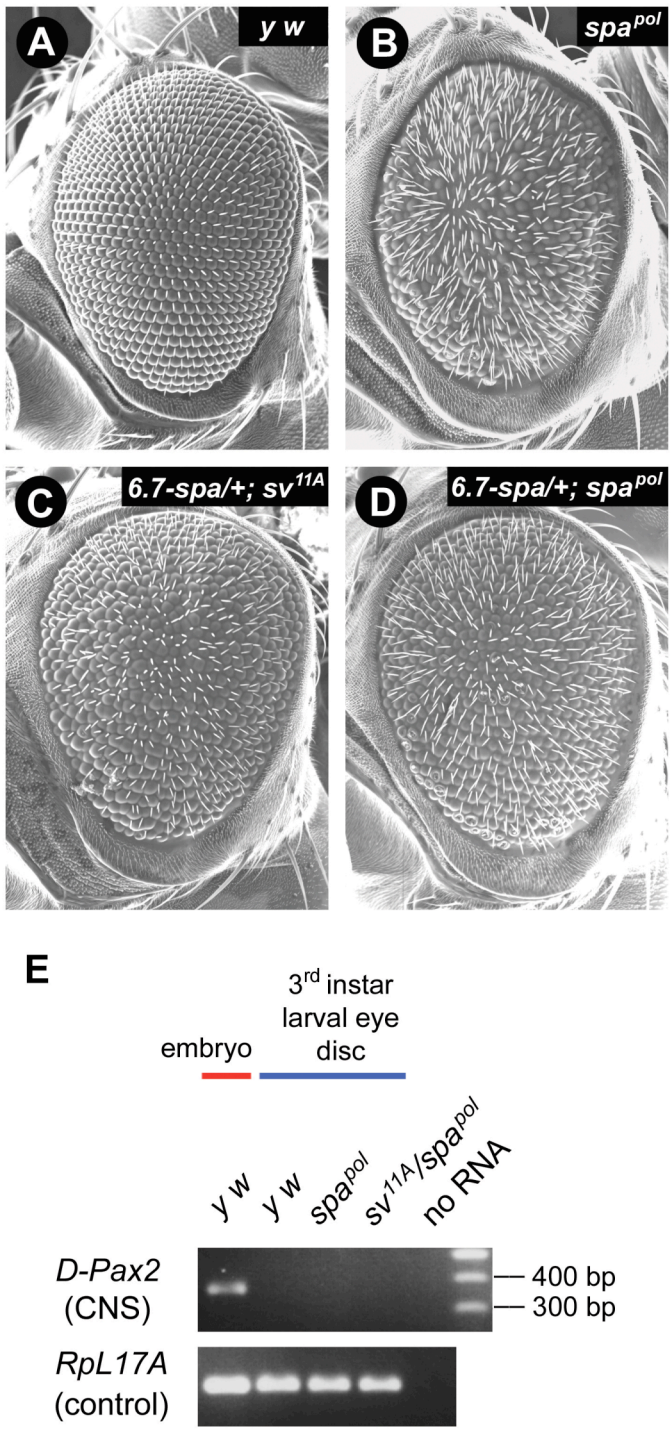

Fig. 4. The *spa* enhancer does not activate the CNS promoter.

(A-D) Scanning electron microscope pictures of eyes of *y w* (A), *y w*; *spa*^{pol} (B), *y w*; *6.7-spa/+*; *sv*^{11A} (C), and *y w*; *6.7-spa/+; spa*^{pol} (D) adults. **(E)** RT-PCR analysis of CNS-specific *D-Pax2* transcripts isolated from embryos and third instar larval eye discs of the indicated genotypes.

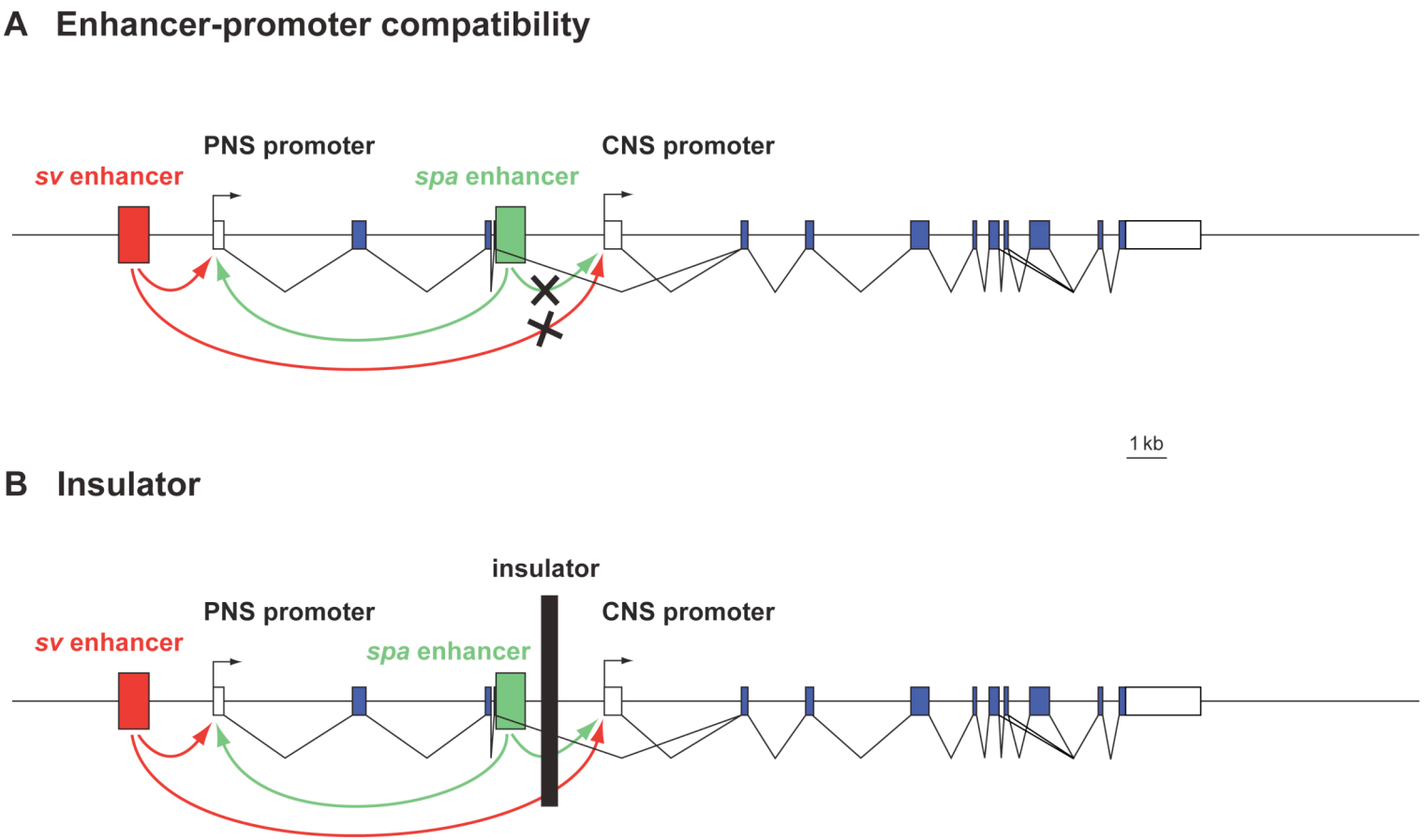

Fig. 5. Model of possible mechanisms regulating the enhancer-promoter specificity at the *D-Pax2* locus.

(A) Enhancer-promoter compatibility: the properties of the enhancer and promoters mediate the transcriptional specificity of *D-Pax2* transcription. **(B)** Enhancer-promoter specificity regulated by an insulator DNA element placed upstream of the CNS promoter.

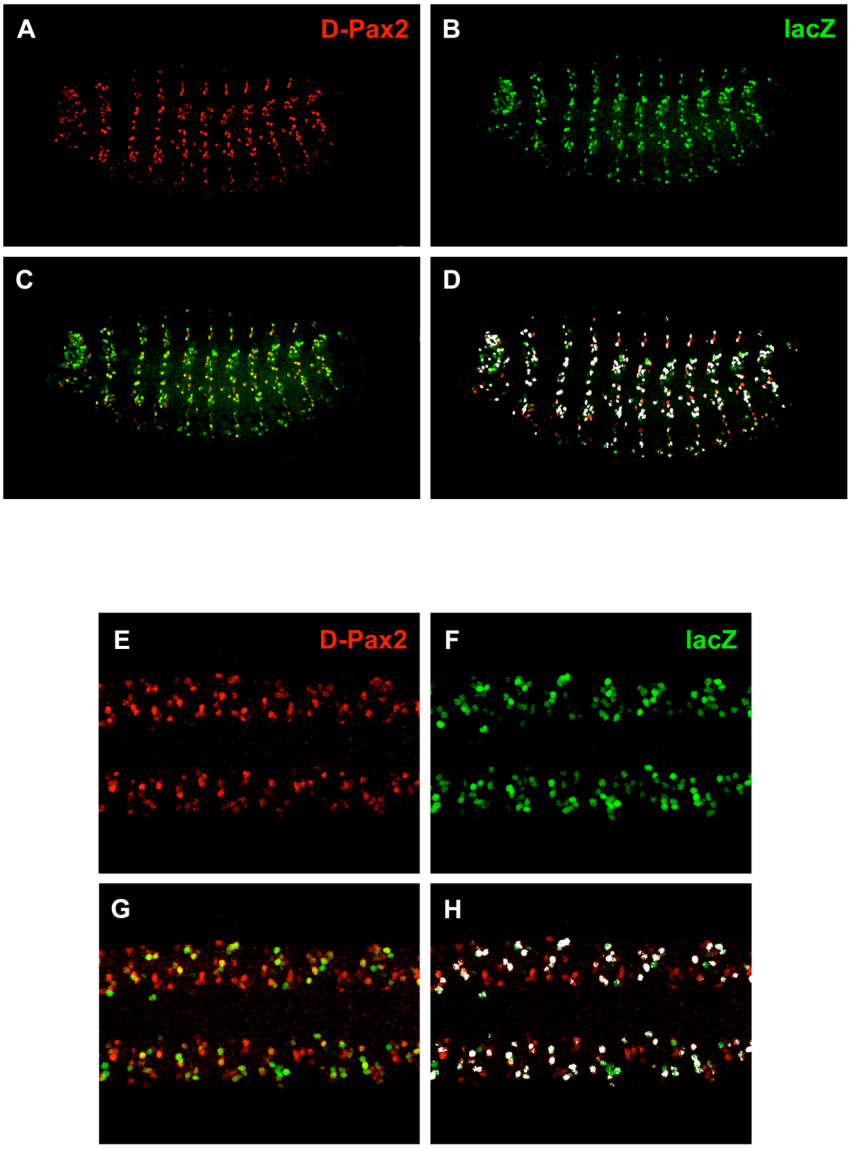

Fig. 6. The *D-Pax2 sv* enhancer and the CNS enhancer can activate transcription from the P-element promoter.

Confocal images of *l(4)2C2* embryos (stage 15) double-stained with anti-D-Pax2 (red) and anti-lacZ (green) antibodies. **(A-D)** Lateral views of embryos, displaying the D-Pax2 expressing cells (red) and LacZ expressing cells (green) in the PNS. Most of the LacZ expressing cells co-localize with the D-Pax2 expressing cells (C). Co-localization was further analyzed by ImageJ software shown as white pixels (D).

(E-H) Confocal images showing the D-Pax2 expressing cells (red) and LacZ (green) expressing cells in the embryonic CNS. Most of the lacZ expressing cells (F) co-localize with the D-Pax2 expressing cells (E). Co-localization is visualized as overlay of the two channels (G) and ImageJ analysis (H).

Chapter 3

Enhancer-specific transvection at the *D-Pax2* locus of *Drosophila melanogaster*

Summary

Transvection is a genetic phenomenon observed when complementation of two different mutant alleles of a gene depends on chromosome pairing. In its simplest model, it is explained as activation of one promoter by the enhancer of the other allele. Transvection is shown to exist at the *D-Pax2* locus. We have demonstrated for the first time that not all enhancers of the *D-Pax2* locus are endowed with this property. The eye-specific *sparkling* (*spa*) enhancer exhibits transvection, whereas the *shaven* (*sv*) enhancer, a PNS-specific enhancer, does not. Our results clearly demonstrate that transvection is specific for the enhancer rather than the promoter.

Introduction

In *Drosophila* homologous chromosomes are always paired in somatic cells (Stevens, 1908; Metz, 1916; Fung et al., 1998). Pairing of homologous chromosomes or regions of chromosomes can exert profound effects on gene expression, collectively termed as *trans*-sensing effects (Tartof and Henikoff, 1991; Henikoff and Comai, 1998). Ed Lewis first coined the term transvection for the phenomenon of pairing-dependent inter-allelic complementation observed at the *Ultrabithorax* (*Ubx*) locus of *Drosophila* (Lewis, 1954). Since then transvection has been reported to occur at several genetic loci, including *Abdominal-B* (*Abd-B*) (Sipos et al., 1998), *apterous* (*ap*) (Gohl et al., 2008), *decapentaplegic* (*dpp*) (Gelbart, 1982), *eyes absent* (*eya*) (Leiserson et al., 1994), *Salivary gland secretion 4* (*sgs4*) (Korge, 1981), *Sex combs reduced* (*Scr*) (Southworth and Kennison, 2002), *Ultrabithorax* (*Ubx)* (Martinez-Laborda et al., 1992), *vestigial* (*vg*) (Coulthard et al., 2005), *yellow* (*y*) (Geyer et al., 1990; Morris et al., 1999), *white*^{speckled} (w^{sp}) (Davison et al., 1985), and *wings-up A* (*wup A*) (Marin et al., 2004). Apart from *Drosophila*, several other organisms, including fungi, plants, and mammals, have shown transvection or transvection-like effects (reviewed in Pirrotta, 1999; Wu and Morris, 1999; Duncan, 2002; Kennison and Southworth, 2002; Sipos and Gyurkovics, 2005).

Inter-allelic complementation by *trans*-sensing effects is observed among three classes of alleles, one with mutations in enhancers, another in promoter or coding regions, and a third in which an insulator has been inserted between enhancers and promoter. Transvection studies with the *y* gene have led to two models that explain gene activation in *trans* that depends on the pairing of homologous genes. In one model, an intact enhancer on one chromosome activates transcription from an intact promoter of the intact homologous transcribed region on the paired chromosome. In the other model, pairing with the homologous gene, whose enhancer and promoter may be deleted, permits the enhancer to bypass the inserted insulator and activate the cognate promoter through a pairing-mediated change in gene structure (Morris et al., 1998; Morris et al., 1999). In addition, it has been demonstrated that the entire *Drosophila* genome is permissive to transvection by the insertion at many locations of transgenes, from which either the promoter or the enhancer has been removed by the Cre or Flip recombinases (Chen et al., 2002). Despite considerable progress over the past 50 years, the molecular mechanism underlying transvection is not well understood. The extent of somatic homologous chromosome pairing and the general permissiveness of the *Drosophila*

genome to transvection, suggest it to be a general phenomenon. However, the number of genetic loci known to exhibit transvection is low. This may result from the fact that only for few genes enhancer mutations are known that could be tested for complementation with structural mutant alleles. Moreover, whether all or only few enhancers are able to activate a paired allele in *trans* is not known.

Here we demonstrate transvection at the *D-Pax2* locus by inter-allelic complementation tests. The *spa* enhancer of *D-Pax2* is able to activate in *trans*, whereas the *sv* enhancer cannot. Since we have shown that both enhancers activate the same of two promoters at this locus (see Chapter 2), this finding suggests that transvection is a property of the enhancer rather than the promoter. To our knowledge, this observation is unprecedented since the discovery of transvection.

Results

Inter-allelic complementation at the *D-Pax2* gene locus

D-Pax2, also known as *shaven* (*sv*) or *sparkling* (*spa*), encodes a paired-domain containing transcription factor (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999). During *Drosophila* development, *D-Pax2* plays a vital role in the development of adult mechanosensory bristles, where it is required for the proper specification and differentiation of the shaft and sheath cells (Fu et al., 1998; Kavaler et al., 1999). In addition, *D-Pax2* plays an important role in eye development, where it is required for the proper specification and differentiation of the primary pigment cells and cone cells (Fu and Noll, 1997; Flores et al., 2000). The *sv* enhancer, which regulates *D-Pax2* transcription specifically in the PNS, has been mapped to a location upstream of the PNS promoter (Fu et al., 1998; Shi, 2001; Chapter 2). The hypomorphic *sv* alleles *shaven-naked* (sv^{n}) and *shaven-depilate* (sv^{de}), which are both insertions of retrotransposons in the *sv* enhancer, display a strong reduction in bristle size or loss of bristles, the *sv* phenotype (Fig. 1B; Fu et al., 1998; Kavaler et al., 1999). By contrast, the 1.6 kb deletion, spa^{pol}, which uncovers the eye-specific *spa* enhancer as well as the third and fourth exons of *D-Pax2* (Fig. 2), produces rough eyes, the *sparkling* (*spa*) eye phenotype (Fig. 1E; Fu and Noll, 1997).

In an EMS-induced mutagenesis screen to identify novel genes regulating eye development (Casci et al., 1999), two mutations were obtained that mapped to the *D-Pax2* locus and were kindly given to us by from Tantia Casci. These *D-Pax2* alleles, *E67* and *E69*, are homozygous lethal and fail to complement the *D-Pax2* hypomorphic alleles sv^{n} and sv^{de} (Fu et al., 1998; Kavaler et al., 1999). By contrast, transheterozygous sv^{n}/*E67* flies displayed the *sv* phenotype (Fig. 1C). Most of the macrochaete bristles on the notum are shaftless, whereas the microchaete bristles have reduced shafts. Sequencing the *D-Pax2* coding regions of the *E67* and *E69* alleles showed point mutations generating an early stop codon in the 4th and 7th exon, respectively (Fig. 2; Erich Frei, personal communication). Thus, *E67* and *E69* are new *sv* point mutation alleles of *D-Pax2*, sv^{E67} and sv^{E69}. Although these failed to complement the *sv* hypomorphic alleles for the *sv* phenotype, surprisingly these alleles complemented the spa^{pol} allele for the *spa* phenotype. Thus, sv^{E67}/spa^{pol} flies showed nearly wild-type eyes (Fig. 1F). This inter-allelic complementation can be explained in two ways: (i) the *spa* enhancer of the *sv* point mutation alleles, sv^{E67} and sv^{E69}, activates transcription from the PNS promoter on the spa^{pol} chromosome in *trans*, or (ii) the *spa* enhancer activates transcription in *cis* from the other *D-Pax2* promoter, the CNS promoter (Fig. 2).

The *spa* enhancer exhibits transvection

To test whether the *spa* enhancer is able to activate transcription from the CNS promoter in *cis*, we performed RT-PCR analysis of *D-Pax2* transcripts produced in third instar eye discs. If the *spa* enhancer is able to activate the CNS promoter, larval eye discs should include CNS-specific transcripts of *D-Pax2*. In eye discs of third instar sv^{11A}/spa^{pol} or *y w* larvae, no CNS-specific *D-Pax2* transcripts were detectable (Fig. 4E of Chapter 2). This shows that the *spa* enhancer cannot activate the CNS promoter and hence suggests that the *spa* enhancer of the *sv* point alleles activates transcription on the homologous gene in *trans*, i.e., exhibits transvection.

To test whether the *spa* enhancer is able to activate transcription in *trans*, we performed a complementation test, using promoter-less and enhancer-less *D-Pax2* alleles. The *D-Pax2* promoter deletion allele sv^{11A} was generated by imprecise excision of the P-element insertion *l(4)2C2*, located upstream of the PNS promoter (Fig. 2). Molecular characterization of sv^{11A} allele revealed a deletion of 2.2 kb extending from -1085 bp to +1175 bp relative to the *D-Pax2* PNS transcription start site. This deletion uncovers the entire *D-Pax2* promoter including the first exon (Fig. 2). Similar to the sv^{A122} deletion allele, homozygous sv^{11A}

animals die as first instar larvae. These animals can be rescued to fertile adults by the *6.7-spa* transgene, expressing *D-Pax2* under the control of the 6.7 kb upstream region spanning the *sv* enhancer (Fu et al., 1998). To test the ability of the *spa* enhancer to support transvection, we tried to complement the *spa* enhancer deletion allele spa^{pol} with the promoter deletion allele sv^{11A}. Indeed, the sv^{11A} allele complemented the spa^{pol} allele, as sv^{11A}/spa^{pol} flies showed nearly wild-type eyes (Fig. 3A). Thus, this inter-allelic complementation test confirms our conclusion that the *spa* enhancer is able to activate in *trans*. Moreover, as we have shown that the *spa* enhancer cannot activate the CNS promoter in *cis*, it follows that the inter-allelic complementation between spa^{pol} and sv^{11A} resulted only from transvection. That this transvection depends on the *spa* enhancer is obvious from the rough eye phenotype of the *spa* enhancer-less spa^{pol} flies.

The *spa* enhancer can activate in *trans*

D-Pax2 mRNAs produced from the spa^{pol} allele in the eye disc are 180 nucleotides shorter than those transcribed from the wild-type allele because they lack the third and fourth exon (Fig. 2). Hence, spa^{pol} transcripts can be easily distinguished from wild-type transcripts, which enables us to identify the chromosome from which *D-Pax2* is transcribed. If inter-allelic complementation between sv^{11A} and spa^{pol} depends on activation by the *spa* enhancer in *trans*, eye discs of third instar sv^{11A}/spa^{pol} larvae are expected to generate *D-Pax2* transcripts only from the spa^{pol} chromosome. To test this, we analyzed *D-Pax2* transcripts by semi-quantitative RT-PCR of total RNA isolated from third instar eye discs and primers located in the 2nd exon (P3) and 5th exon (P4) (Fig. 3B). Indeed, only spa^{pol}-specific transcripts were observed in sv^{11A}/spa^{pol} eye discs, whereas in controls of *y w* or spa^{pol} eye discs only wild-type or no transcripts were detected (Fig. 3B). These results corroborate at the molecular level that the *spa* enhancer of the sv^{11A} allele only activates its cognate PNS promoter that is present in *trans* on the spa^{pol} chromosome. Therefore, transvection at the *D-Pax2* locus is possible through the *spa* enhancer.

Activation by *spa* enhancer shows no preference for promoter in *cis*

It has been shown that the body and wing enhancers of the *y* locus exhibit transvection but also a strong preference for the promoter in *cis* (Morris et al., 2004; Lee and Wu, 2006). In other words, these enhancers activate in *trans* only in the absence of an intact cognate promoter in *cis*. Therefore, we wondered whether the *spa* enhancer exhibits such preference for its cognate promoter in *cis* as well. To test this, *D-Pax2* transcripts produced in $spa^{pol}/+$

eye discs were analyzed by RT-PCR as described above. In this heterozygous combination of the spa^{pol} allele, the *spa* enhancer on the wild-type chromosome is encountered with its cognate promoter in *cis* and another in *trans* located on the spa^{pol} chromosome. Surprisingly, these spa^{pol}/+ eye discs produced wild-type and spa^{pol}-specific transcripts in about equal amounts (Fig. 3B). This suggests that the *spa* enhancer on the wild-type chromosome can activate transcription from both cognate promoters in *cis* and in *trans*. In summary we conclude that, unlike the enhancers of the *y* gene, the *spa* enhancer shows no preference for its cognate promoter in *cis*.

The *spa* enhancer cannot activate in *trans* when not paired with its promoter

Classical transvection depends on allelic pairing (Lewis, 1954). The *D-Pax2* gene is located on the small 4th chromosome of *Drosophila*. Because of its relatively short length, it is difficult to disrupt its pairing by translocations. Nevertheless, we could test the dependency of transvection at the *D-Pax2* locus on pairing in a spa^{pol} background indirectly by a *D-Pax2* transgene that included the *spa* enhancer. The *D-Pax2 CNS-res-2* transgene (Chapter 4), which consists of a 23 kb genomic *D-Pax2* fragment extending from, and including, the *spa* enhancer to the next gene downstream of *D-Pax2*, did not rescue the spa^{pol} eye phenotype (Fig. 4A). Similarly, the *spa-0.95-lacZ*, in which the *lacZ* coding region is placed under the control of the *spa* enhancer and PNS promoter (Shi, 2001), did not rescue the spa^{pol} eyes (Fig. 4B). This suggests that the *spa* enhancer cannot activate its promoter on the 4th chromosome in *trans* when present elsewhere in the genome. Therefore, to exert transvection the *spa* enhancer has to be in close proximity to the promoter present in *trans*, as is typical for transvection. Most cases of transvection depend on the normal *zeste* (*z*) function (Duncan, 2002; Kennison and Southworth, 2002). We have tested whether transvection of the *spa* enhancer also depends on *z*. Indeed, sv^{11A}/spa^{pol} flies in *z* mutant background (z^a or z^1) showed a rough phenotype in the posterior part of the eye but did not abolish completely rescue by transactivation (Fig. 4C,D), which implies that transvection through the *spa* enhancer is only partially dependent on the *z* function.

The *sv* enhancer may support transvection at a very low level

Like the *spa* enhancer, the *sv* enhancer activates transcription specifically from the PNS promoter (Chapter 2). Therefore, we tested whether the *sv* enhancer also supports transvection by an attempt to complement the promoter deletion allele sv^{11A} with the hypomorphic *sv* alleles, sv^{de} and sv^{n}. If the intact *sv* enhancer on the sv^{11A} allele is able to

activate transcription from the PNS promoter located on sv^{de} or sv^{n} allele, the *sv* phenotype should be suppressed. Surprisingly, no complementation was observed although the bristle phenotype of sv^{11A}/sv^{de} flies is slightly weaker than that of sv^{de} flies (Fig. 5A,B), whereas no significant difference is observed between the phenotypes of sv^{11A}/sv^{n} and sv^{n} flies (Fig. 5C,D). This suggests that the *sv* enhancer supports a low level of transvection, much lower than that of the *spa* enhancer, with the PNS promoter. The fact that this low level of transvection is only recognized with the sv^{de}, but not the sv^{n}, allele is probably explained by the accumulation of modifiers in the sv^{n} stock, whereas sv^{de} mutants cannot be maintained as homozygous stock. Since we cannot rule out that the insertions of retrotransposons in the sv^{n} and sv^{de} alleles prevent pairing with other *D-Pax2* alleles and thus transvection, it is possible that the *sv* enhancer of sv^{11A} supports stronger transvection if combined with a *sv* allele that lacks the *sv* enhancer but not its cognate PNS promoter.

Hence, as for the *spa* enhancer, we tried to investigate whether the *sv* enhancer is able to support transvection by testing deletion alleles of the PNS promoter or *sv* enhancer for complementation. To perform this test, a *sv* enhancer deletion allele, sv^{X14P}, was generated by imprecise excision of the P-element insertion *l(4)2C2* (Fig. 2). The sv^{X14P} allele contains a deletion of 1589 bp, from -2982 to -1394, which removes most of the previously mapped *sv* enhancer (Shi, 2001). Unlike sv^{11A} homozygotes, which show a complete loss of *D-Pax2* function in the PNS and hence die as first instar larvae, 36% of sv^{X14P} homozygotes survive to adulthood. However, these flies cannot walk and die soon after eclosion. Like other hypomorphic *sv* mutants, homozygous sv^{X14P} flies displayed a strong *sv* phenotype (Fig. 6B), which implies that most but not all of the *sv* enhancer is deleted in the sv^{X14P} allele. Most macrochaete bristles on the notum lack the shaft structures and instead exhibit double socket cells (Fig. 6B). Transformation of shaft to socket cells is characteristic for the phenotype of hypomorphic *sv* alleles (Kavaler et al., 1999). Similarly, the microchaete bristles on the notum are absent or have greatly reduced shafts (Fig. 6B), as compared to sv^{+} flies (Fig. 6A), while all eye bristles are missing (Fig. 7A,B).

To test whether the *sv* enhancer deletion allele complements the PNS promoter deletion allele, we combined sv^{X14P} with sv^{11A}. If the *sv* enhancer on the sv^{11A} chromosome is able to activate transcription from its cognate promoter on the sv^{X14P} chromosome, then these two alleles should complement each other. However, like sv^{de}, sv^{X14P} did not complement with sv^{11A}, but showed a slight rescue of the sv^{X14P} bristle phenotype that was more easily

recognized when compared to that of hemizygous $sv^{X14P}/Df(4)G$ flies (Fig. 6B-D). Only 31% of sv^{X14P}/sv^{11A} flies survived and most of them died immediately after eclosion, similar to homozygous sv^{X14P} flies. The sv^{X14P}/sv^{11A} flies showed a strong *sv* phenotype (Fig. 6C), very similar to that of sv^{X14P} flies (Fig. 6B). The notal macrochaete bristles have no shaft structures, and a double socket phenotype is observed (Fig. 6C). Most of the microchaete bristles on the notum showed a minimal increase in length compared to sv^{X14P} flies (Fig. 6B,C). Also the eye bristles were not rescued (Fig. 7C). However, one copy of sv^{X14P} tested over *Df(4)G*, a large deficiency on the 4th chromosome uncovering the *D-Pax2* locus along with several neighboring genes (Fu et al., 1998), showed only 7% survival to adults, all of which died after eclosion. These dead flies exhibited a strong *sv* phenotype (Figs. 6D and 7D), similar to but stronger than that of sv^{X14P} flies (Figs. 6B and 7B). In conclusion, these results demonstrate that PNS promoter deletion allele sv^{11A} does not complement with the *sv* enhancer deletion allele sv^{X14P} but slightly rescues the bristle phenotype of sv^{X14P} and hence seems to support a very low level of transvection.

The *sv* enhancer may be able to activate in *trans* at a low level

To obtain a quantitative measure for the ability of the *sv* enhancer to activate in *trans*, we relied on quantitative real-time PCR (qPCR) analysis for quantification of PNS-specific *D-Pax2* mRNA levels in embryos (Fig. 8A) and early pupal wing discs (Fig. 8B) of wild-type and mutant larvae. Homozygous or transheterozygous mutant embryos or pupae were selected by screening for the absence of a 4th-chromosome marked with actin-GFP. Quantitative RT-PCR was performed with total RNA isolated from embryos or white pupal wing discs and PNS-specific primers (P1 and P2 in Fig. 2). As internal controls we have quantified the CNS-specific *D-Pax2* transcripts in embryos (using C3 and C4 primers in Fig. 2) and *senseless* (*sens*) transcripts in white pupal wing discs. All reactions were performed once (white pupal wing discs) or twice (embryos) in triplicates, and transcript values were normalized to transcript levels of three constitutively active genes and set at 1.0 in *y w* embryos or pupal wing discs. As expected, only background levels of PNS-specific *D-Pax2* transcripts were detectable in sv^{11A} embryos (Fig. 8A). By contrast, in sv^{X14P} embryos the level of PNS-specific transcripts was reduced to 39% of that in *y w* control embryos. In sv^{X14P}/sv^{11A} embryos, the level of PNS-specific mRNA was reduced to 31%, which is only 5% above that of hemizygous $sv^{X14P}/Df(4)G$ embryos (Fig. 8A). Thus, the PNS-specific *D-Pax2* mRNA level in sv^{X14P}/sv^{11A} embryos is only slightly higher than that produced by one copy of sv^{X14P}, which confirms that transvection contributes relatively little to *D-Pax2* levels

in the PNS of sv^{X14P}/sv^{11A} embryos. Similarly, we have measured *D-Pax2* mRNA levels in white pupal wing discs. Surprisingly, here *D-Pax2* transcript levels were about two fold higher in sv^{X14P}/sv^{11A} than in $sv^{X14P}/Df(4)G$ discs (Fig. 8B). However, contrary to what is expected discs with two copies of sv^{X14P} show lower transcript levels than discs with one copy in $sv^{X14P}/Df(4)G$ white pupae (Fig. 8B). This result casts doubt on the precision by which these transcript levels can be measured by this method. A more reliable, though not quantitative, test therefore is the bristle phenotype. We conclude that the *sv* enhancer is able to activate in *trans* minimally which, unlike the *spa* enhancer, is not sufficient to rescue the survival and the *sv* phenotype in complementation tests.

Finally, we have tested whether the expression pattern of *D-Pax2* in the embryonic PNS can be rescued by the *sv* enhancer acting in *trans*. *D-Pax2* is expressed in the developing PNS, which includes all developing external sensory organs and the chordotonal organs (Fig. 9A; Fu et al., 1998; Kavaler et al., 1999). As expected, D-Pax2 expression is completely abolished in the PNS of sv^{11A} embryos (Fig. 9B), whereas in sv^{X14P} embryos, the number of D-Pax2 expressing cells is reduced compared to wild-type embryos (Fig. 9A,C). Clearly, the lateral chordotonal cell clusters do not express D-Pax2 in sv^{X14P} embryos (Fig. 9C). Similarly, sv^{X14P}/sv^{11A} embryos fail to express *D-Pax2* in chordotonal organs, and the PNS expression pattern is similar to that of sv^{X14P} embryos (Fig. 9D). Thus, these results suggest that the *sv* enhancer cannot rescue expression in the embryonic PNS by activation of *D-Pax2* in *trans*, although small effects cannot be ruled out. We conclude that, in contrast to the *spa* enhancer, the *sv* enhancer may support transvection only at a very low level. Therefore, transvection is a quantitative property of the enhancer.

Discussion

In the present study, we report a new example of transvection in *Drosophila* at the *D-Pax2* locus. We found that the *spa* enhancer of *D-Pax2* supports transvection to the extent that the eye phenotype is rescued solely by transvection, whereas the *sv* enhancer of the same locus is unable to rescue the *sv* phenotype by transvection, which suggests that transvection is an enhancer-specific phenomenon. Thus, two different tissue-specific enhancers of a single gene locus, activating the same cognate promoter in *cis*, exhibit contrasting abilities to activate in *trans*. Hence, this property is enhancer- rather than promoter-specific.

We have shown that the deletion allele of the *spa* enhancer complements that of the PNS promoter. Therefore, the inter-allelic complementation results from the ability of the *spa* enhancer to activate a promoter in *trans*, as proposed for transvection of the *y* gene (Morris et al., 1998; Morris et al., 1999). Taking advantage of the shorter *D-Pax2* transcripts of the spa^{pol} allele, we have shown that the *spa* enhancer is able to activate transcription from its cognate promoter in *cis* as well as in *trans*. This is different from the *y* gene, where the body and wing enhancers show a strong preference for a promoter in *cis* to one in *trans*. Our results, however, are consistent with a previous report (Goldsborough and Kornberg, 1996). In addition, these authors demonstrated that the chromosomal pairing stimulates transcription in both *cis* and *trans* because disruption of pairing leads to a decrease in transcription of both alleles (Goldsborough and Kornberg, 1996).

The *sv* enhancer deletion allele, sv^{X14P}, failed to complement the PNS promoter deletion allele, sv^{11A}, for survival and bristle phenotype although a low level of transvection was detectable. Similarly, quantitative measurements of the *D-Pax2* transcripts revealed that the *sv* enhancer is able to show weak activation in *trans*. We propose that the non-complementation between the *sv* enhancer deletion allele and the PNS promoter deletion allele results from insufficient *D-Pax2* transcript levels produced through enhancer *trans*-activation. By contrast, in the case of transvection by the *spa* enhancer, *D-Pax2* transcript levels produced in *trans* are sufficient to rescue the eye phenotype in inter-allelic complementation tests. Thus, the ability to support transvection depends on the quantitative property of the enhancer. In summary, our data strongly suggest that transvection is a property of the enhancer but not of the promoter because only one of two different enhancers activating the same promoter in *cis* can activate it also in *trans*. Hence transvection at the *D-Pax2* locus depends on the property of the individual enhancer.

Materials and methods

Fly stocks

The following fly stocks were used. *y w,* sv^{n}, sv^{de}, ci^{D} spa^{pol}, *Df(4)G* (Fu, et al., 1998), $sv^{\Delta 122}$ (Shi, 2001), $sv^{\Delta 122\text{-}P}$, sv^{E67}, sv^{E69}, sv^{11A}, sv^{X14P}, *spa-0.95-lacZ* (Shi, 2001), *l(4)2C2* (kindly provided by J. Kronhamn and A. Rasmuson-Lestander), z^{a}, z^{1} (stocks BL-1059 and BL-200

from Bloomington Stock Center), *P{ActGFP}unc-13*^{GJ} (GFP-marked 4th-chromosome, stock BL-9549 from Bloomington Stock Center).

Generation of *D-Pax2* mutants

The *D-Pax2* alleles *sv*^{11A}, *sv*$^{\Delta 122}$, *sv*$^{\Delta 122\text{-}P}$ were generated by imprecise excisions of P-elements (see Materials and methods of Chapter 2). The *sv* enhancer deletion allele, *sv*^{X14P}, was generated by imprecise excision of the *w*$^{+}$ *PlacW* P-element insertion *l(4)2C2* (Fig. 1). The animals in which the P-element is mobilized with the *Δ2–3* P-element transposase (*w*; *TMS, P{Δ2-3}99B/+*; *l(4)2C2/spa*^{pol}) were crossed to *spa*^{pol}/*ci*^{D} *spa*^{pol} flies. F1 males with *spa*$^{+}$ but white eyes of the genotype *w*; *TMS, P{Δ2-3}99B/+*; *ex(4)2C2/ci*^{D} *spa*^{pol} were crossed to *Df(4)G/ci*^{D} *spa*^{pol} virgins. In these males the P-element has been excised and not only its *w*$^{+}$ marker silenced, as silenced *w*$^{+}$ markers would result in red mosaic eyes because of the continued presence of the P-element transposase. In 7 out of over 100 such males the P-element had not excised cleanly because they produced no *ex(4)2C2/Df(4)G* offspring. Rather the excision created a lethal PNS expression mutation of *D-Pax2* at or near the original P-element insertion site, which is uncovered by the deficiency *Df(4)G*, which also deletes neighboring genes (Fu et al., 1998). The lethal excisions could delete the *D-Pax2* PNS promoter and/or the *sv* enhancer, but not also the *spa* enhancer since the F1 males were *spa*$^{+}$, as are their *ex(4)2C2/ci*^{D} *spa*^{pol} offspring used to establish the 7 stocks, in two of which the mutation was characterized in detail. One allele, *sv*^{X4P} is a PNS promoter deletion smaller than in *sv*^{11A} (Fig. 2). The *sv*^{X14P} allele is a 1589 bp deletion from -2982 to -1394 upstream of the PNS transcription initiation site, which surprisingly is 328 bp upstream of the original P-element insertion site and thus deletes more than the *sv* enhancer mapped by *lacZ* reporter genes and *sv* rescue transgenes (Shi, 2001). In addition, the *PlacW* was deleted during mobilization except for 16 bp at its left and 13 bp at its right end. In other words, 29 bp of the inverted repeat DNA and 8 bp target site duplication (in total 45 bp) are located between -1065 and -1074.

RNA isolation

Total RNA was isolated from *Drosophila* embryos, the eye part of eye-antennal discs of wandering third instar larvae, and white pupal wing discs of wild type and *D-Pax2* mutants. To recognize homozygous or transheterozygous *sv* mutants during development, a GFP-marked, *P{ActGFP}unc-13*^{GJ}, 4th chromosome was used as balancer chromosome. Total RNA was isolated with Nucleo Spin RNA II (MACHEREY NAGEL) kit according to the

manufacturer's instructions.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

All enzymatic reactions of RT-PCR were performed with 100 ng of total RNA isolated from eye discs with the One Step RT-PCR kit (Qiagen) in one tube. After reverse transcription, in which first strand cDNA is primed and synthesized with the specific reverse primer for 30 min at 50°C, the HotStarTaq DNA polymerase is activated for 15 min at 95°C. Subsequently, 32 PCR cycles are performed with 1 min denaturation at 94°C, 1 min annealing at 57°C, and 1 min extension at 72°C. The last cycle is followed by a 10 min incubation at 72°C. The DNA products were analyzed on 1.5% agarose gels. The following primers were used in RT-PCR reactions.

D-Pax2 PNS forward (P3): 5'-GATATACAGACATCGACACATC-3',

D-Pax2 PNS reverse (P4): 5'-GACGTGATATGTCACATGGGCGGAC-3'.

PCR reactions with *RpL17A*, a constitutive ribosomal protein gene in *Drosophila* that was used as loading control, used the following primers:

RpL17A forward: 5'-TGATGAACTGTGCCGACAAC-3',

RpL17A reverse: 5'-TGCATTGGATGCAATACGGG-3'.

Quantitative real-time polymerase chain reaction (qPCR)

First-strand cDNA was primed with oligo-dT primer on 0.5 µg of total RNA and synthesized, according to the manufacturer's instructions, by the use of the SuperScript™ III First-strand synthesis kit for RT-PCR (Invitrogen). The generated cDNA pools were used as templates for PCR reactions performed in triplicates for each primer pair. The products were quantified with an ABI 7900HT sequence detection apparatus, which measures the amount of double stranded DNA with SYBR Green fluorescence after every PCR cycle. PCR reactions were made with kits from either ABI (Power SYBR Green PCR Master Mix) or Eurogentech (MESA GREEN qPCR MasterMix Plus). Normalization was based on the transcript levels of three constitutively expressed genes, *actin5C*, *TBP* (TATA-binding protein gene), and *α-tubulin*, in all the RNA samples from the different genotypes. The following primers were used in qPCRs.

D-Pax2 PNS forward (P1): 5'-CAGGGCAGTATTTCGGGTGAT-3',

D-Pax2 PNS reverse (P2): 5'-GTGGGCGACCATTAACGAAT-3',

D-Pax2 CNS forward (C1): 5'-AACCAGAACATGATGGAATACTACACAT-3',

D-Pax2 CNS reverse (C2): 5'-TGGGCGACCATTAACGAATAC-3',

sens forward: 5'-CCAATATTGTGGCAAGCGGT-3',

sens reverse: 5'-GGTGCACTTGTGTGGCTTCT-3',

actin5C forward: 5'-GCCCATCTACGAGGGTTATGC-3',

actin5C reverse: 5'-AATCGCGACCAGCCAGATC-3',

TBP forward: 5'-CGCGCATCATCCAAAAGC-3',

TBP reverse: 5'-GCCGACCATGTTTTGAATCTTAA-3',

α-tubulin forward: 5'-GCCAGATGCCGTCTGACAA-3', and

α-tubulin reverse: 5'-AGTCTCGCTGAAGAAGGTGTTGA-3'.

Immunohistochemistry and microscopy

Embryos were fixed and stained as described previously (Fu and Noll, 1997). Rabbit anti-D-Pax2 antiserum was used as described (Fu and Noll, 1997). Anti-D-Pax2 staining was enhanced by the use of a TSA^{TM} kit (Invitrogen). Confocal microscopy was carried out with a LEICA TCS SP microscope. Images were processed with Adobe Photoshop 7.0 and ImageJ software.

Scanning electron microscopy of adult eyes and nota

Flies are killed by exposure to ether vapors for 5 min and mounted untreated in a JEOL JSM-6360 LV scanning electron microscope. After at least one minute in the vacuum chamber, micrographs of eyes were taken at 220x magnification and of nota at 100x or 85x magnification.

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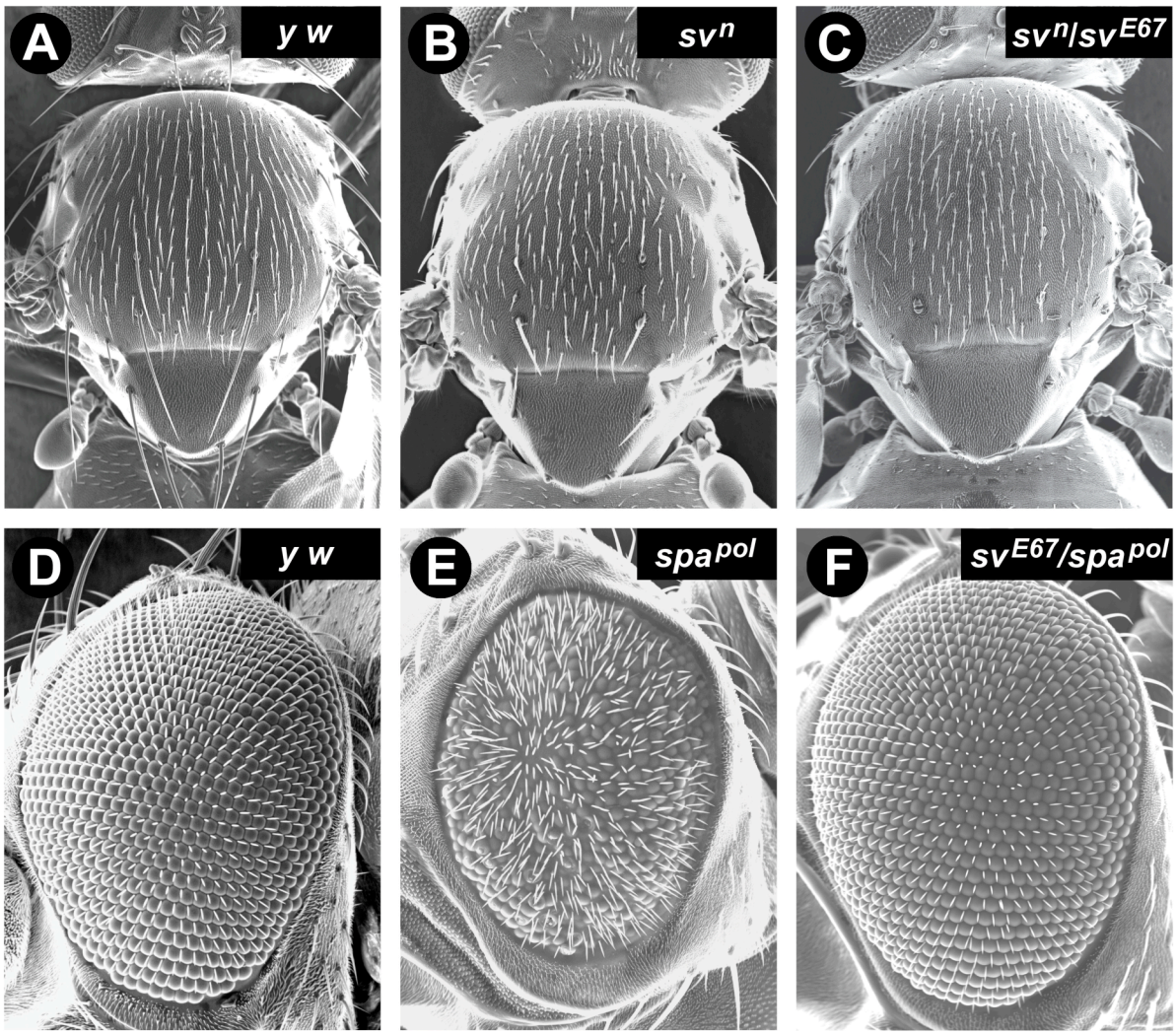

Fig. 1. Interallelic complementation at the *D-Pax2* locus.

(A-F) Scanning electron micrographs of notum and scutellum (A-C) and of compound eyes (D-F) of *y w* (A,D), sv^{n} (B), sv^{n}/sv^{E67} flies (C), spa^{pol} (E), and sv^{E67}/spa^{pol} (F) flies.

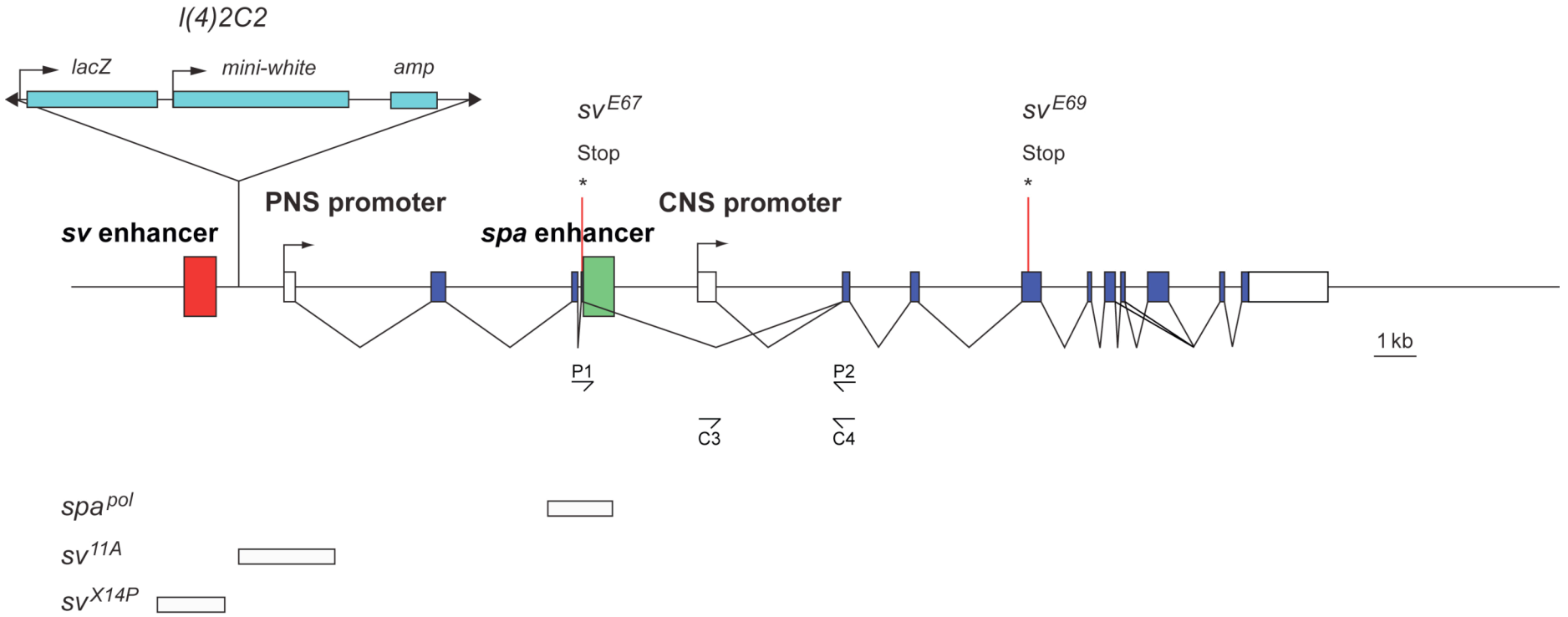

Fig. 2. Molecular and genetic map of the *D-Pax2* locus.

A map of the *D-Pax2* locus on the 4th chromosome is shown with the locations of its two promoters, the PNS and CNS promoter, and transcription start sites (arrows), of its exons (boxes with coding regions in blue), and of its *sv* (red box) and *spa* (green box) enhancers. Below, the deleted regions of deficiency alleles designated on the left are indicated by open boxes. The location of the P-element insertion *l(4)2C2* and a map of its genes are also shown.

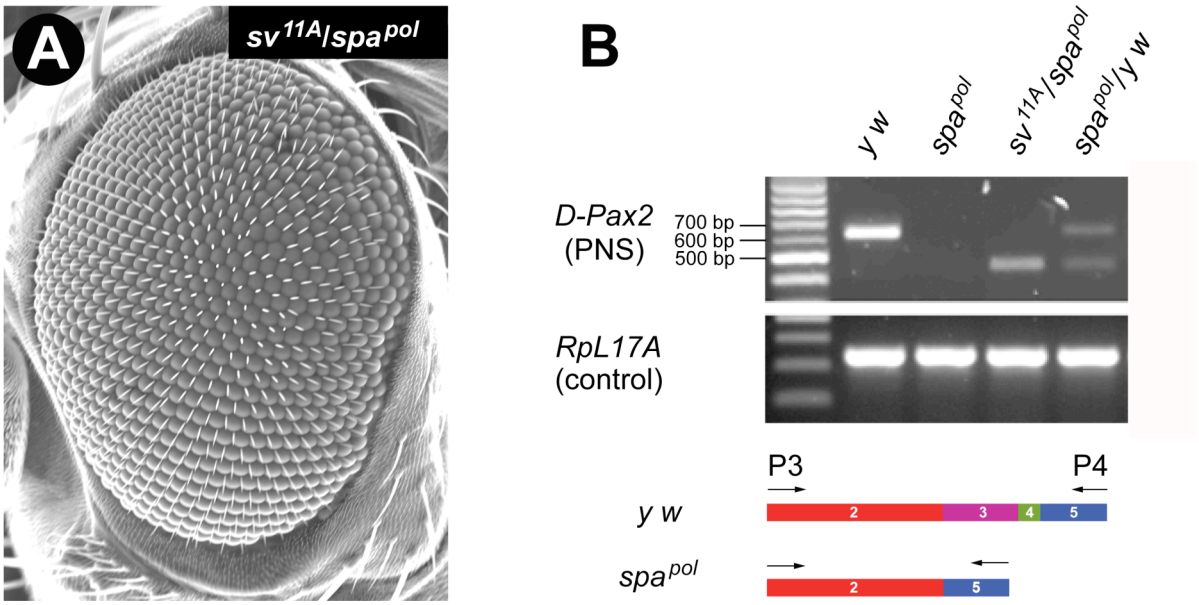

Fig. 3. The *spa* enhancer of *D-Pax2* exhibits transvection.

(A) Scanning electron micrographs of left eye of sv^{11A}/spa^{pol} fly. **(B)** Semi-quantitative RT-PCR analysis of *D-Pax2* transcripts produced from total RNA of 3rd instar larval eye discs. The relevant portions of 1.5% agarose gels illustrating the DNA bands produced by RT-PCR from total RNA of eye discs of indicated genotypes are shown. Below, the *D-Pax2* exons spanned by the PCR reaction with primers P3 and P4 of *y w* and spa^{pol} DNA are indicated.

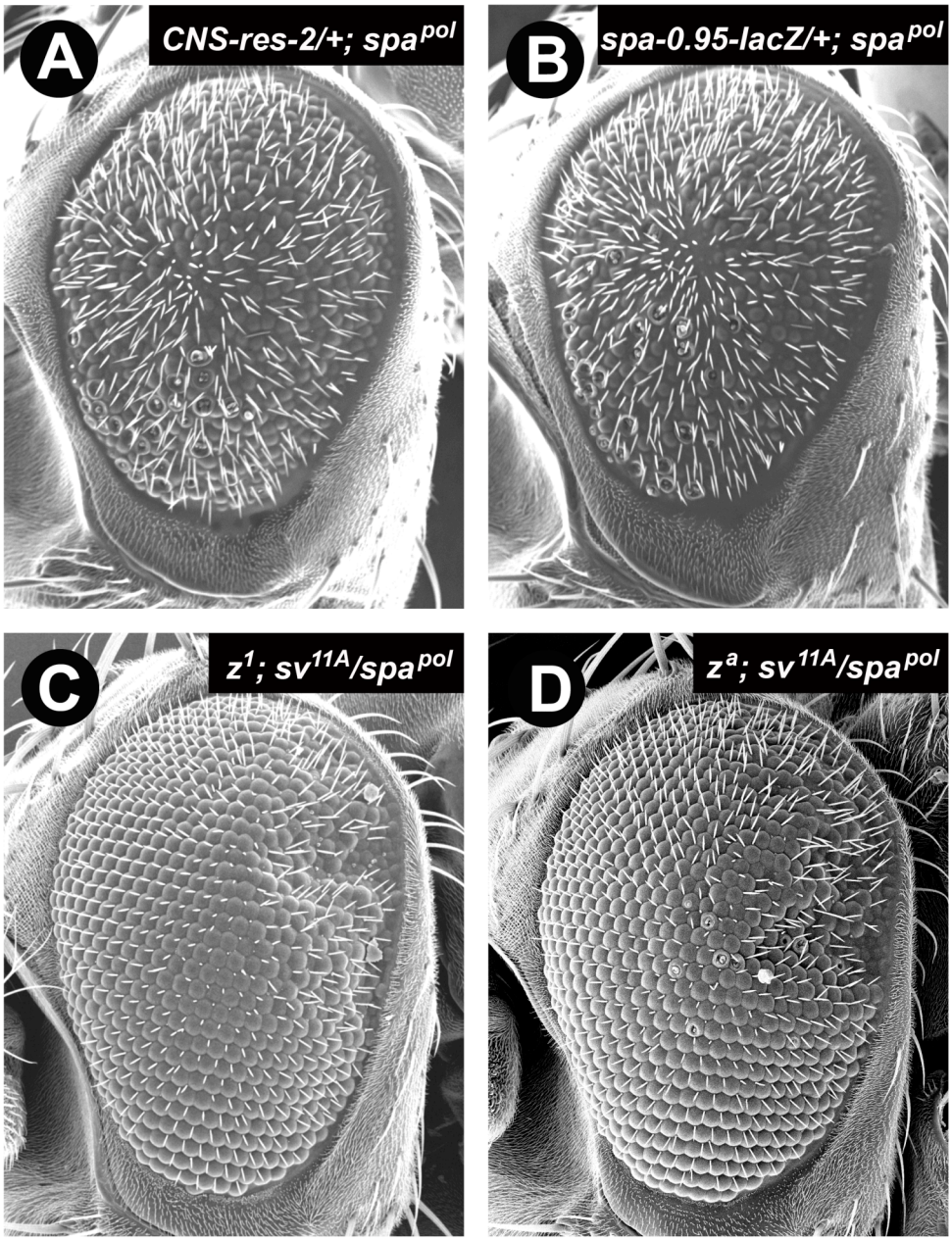

Fig. 4. Transvection by the *spa* enhancer depends on paired alleles and partially on the function of *zeste*.

(A-D) Scanning electron micrographs of *CNS-res-2/+*; spa^{pol} (A), *spa-0.95-lacZ/+*; spa^{pol} (B), z^1; sv^{11A}/spa^{pol} (C), z^a; sv^{11A}/spa^{pol} (D) adult left eyes.

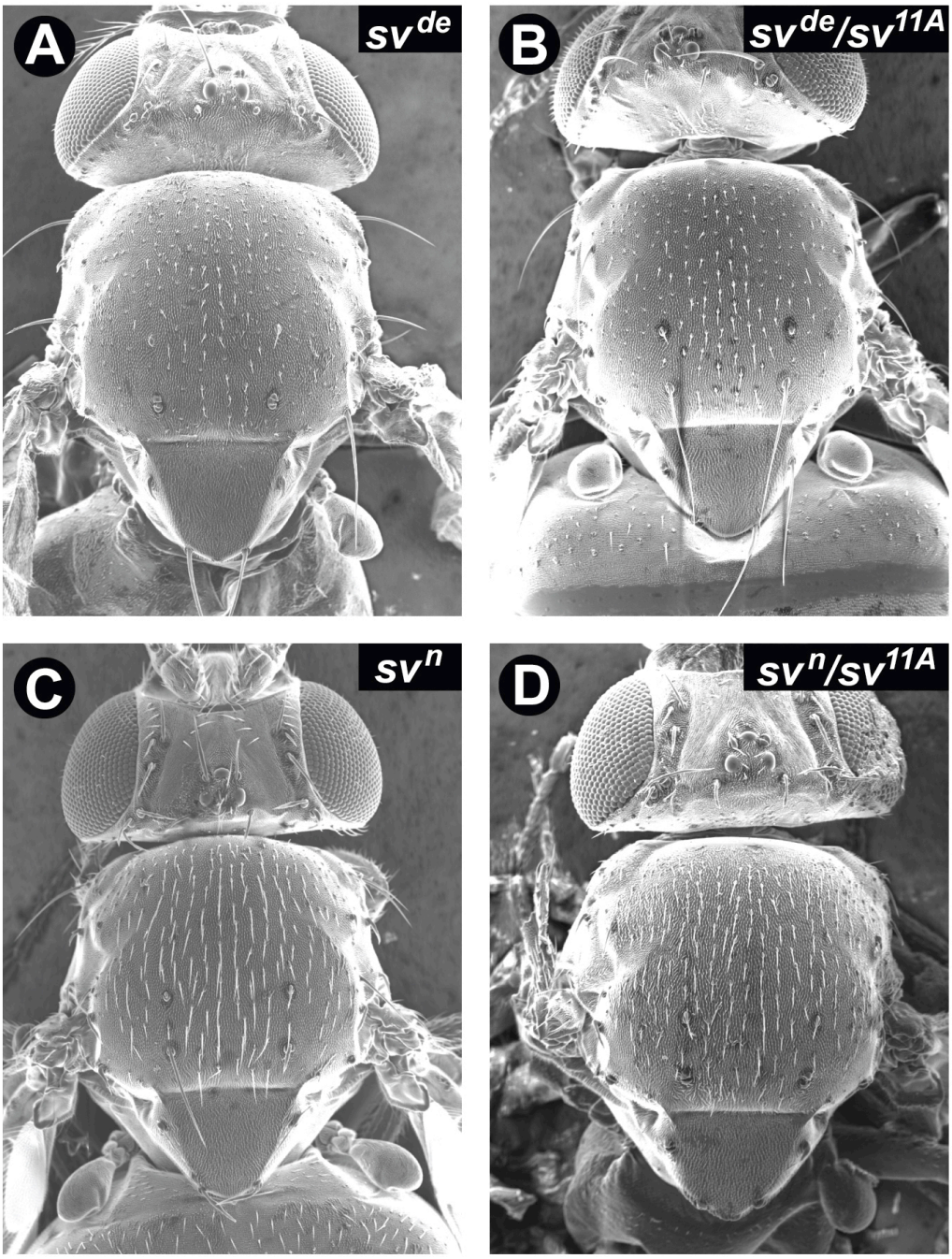

Fig. 5. The promoter deletion allele, *sv*^{11A}, complements the classical *sv* enhancer insertion alleles, *sv*^{de} and *sv*^{n}, at most very little.

(A-D) Scanning electron micrographs of notum and scutellum of *sv*^{de} (A), *sv*^{de}/*sv*^{11A} (B), *sv*^{n} (C), and *sv*^{n}/*sv*^{11A} (D) flies.

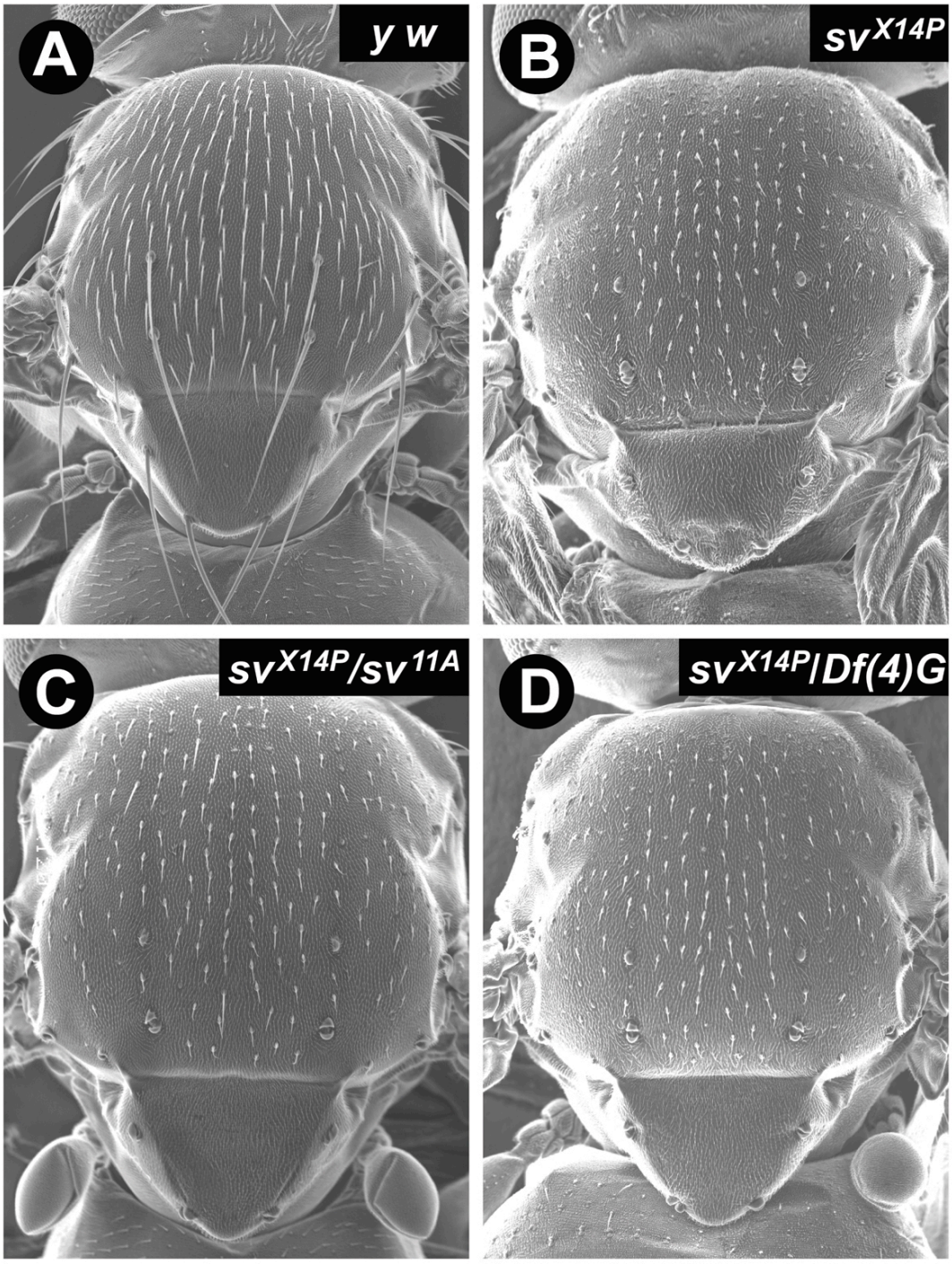

Fig. 6. The promoter deletion allele, sv^{11A}, complements the deletion allele of the *sv* enhancer, sv^{X14P}, for body bristle formation at most very little.

(A-D) Scanning electron micrographs of notum and scutellum of *y w* (A), sv^{X14P} (B), sv^{X14P}/sv^{11A} (C), and $sv^{X14P}/Df(4)G$ (D) flies.

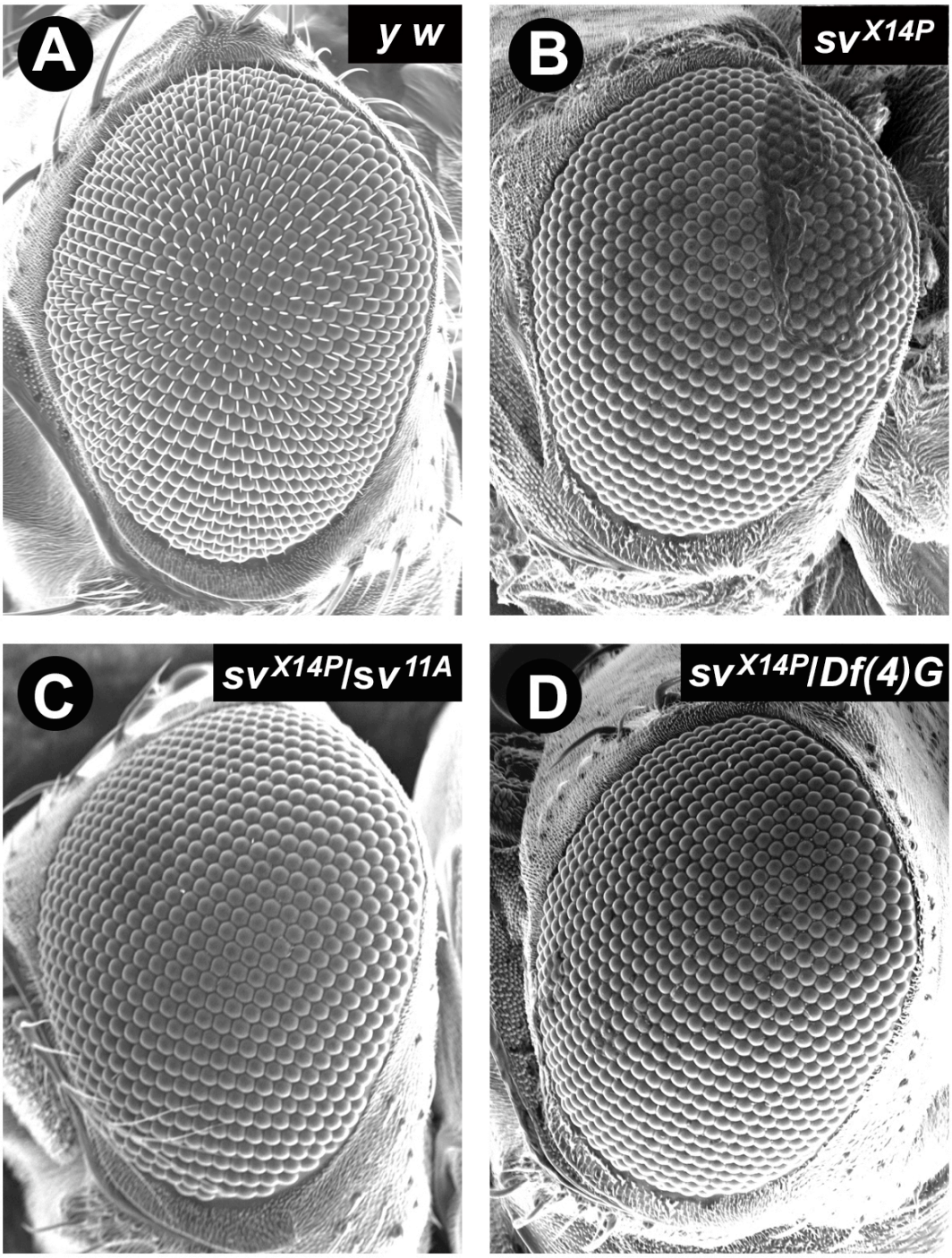

Fig. 7. The promoter deletion allele, sv^{11A}, does not complement the deletion allele of the *sv* enhancer, sv^{X14P}, for eye bristle formation.

(A-D) Scanning electron micrographs illustrating the bristle phenotype of *y w* (A), sv^{X14P} (B), sv^{X14P}/sv^{11A} (C), and $sv^{X14P}/Df(4)G$ (D) eyes.

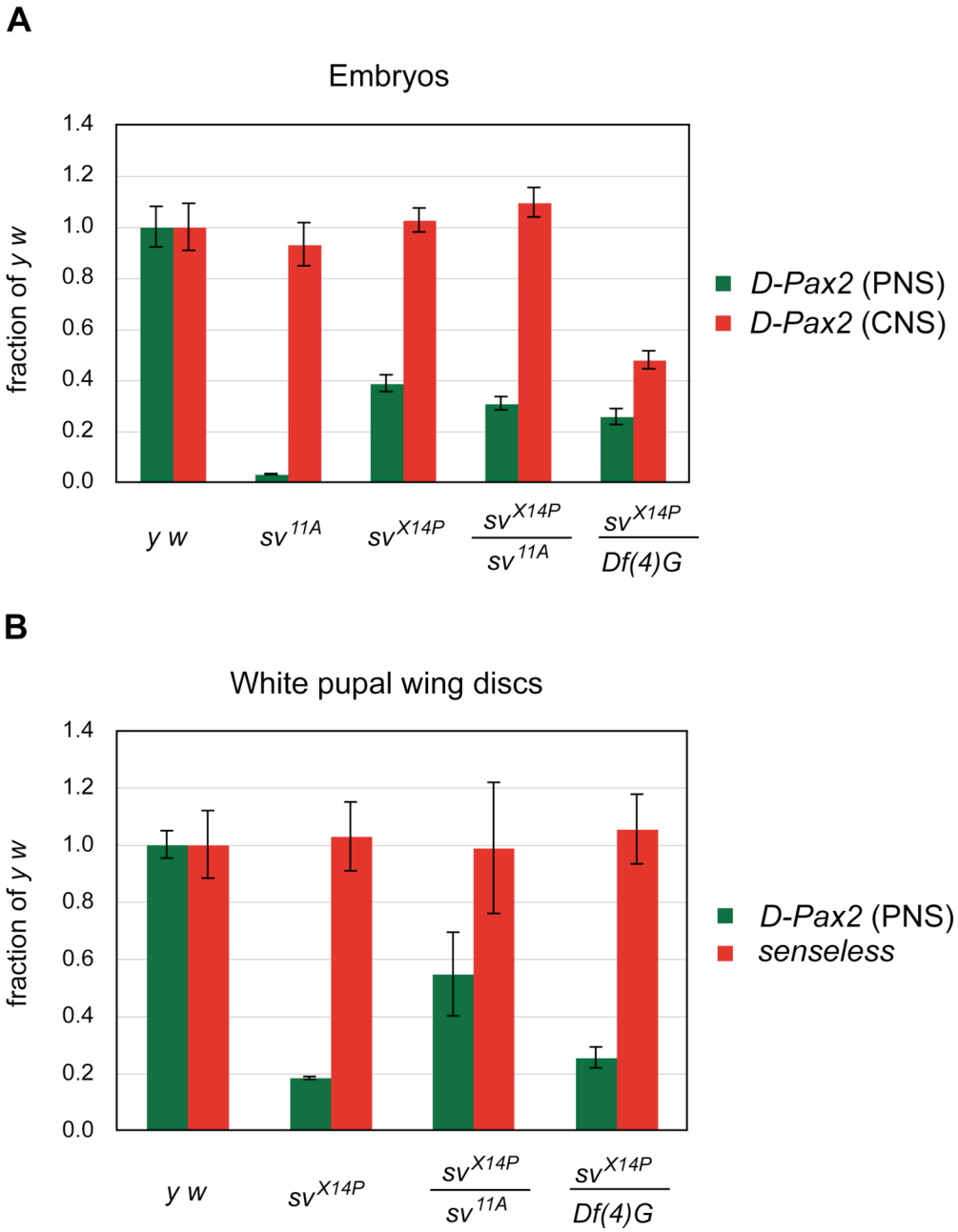

Fig. 8. Quantitative real-time PCR analysis of *D-Pax2* transcripts.

(A,B) qPCR analysis of reverse transcribed PNS-specific transcripts in embryos (A) and white pupal wing discs (B) of genotypes indicated. Error bars represent standard deviations.

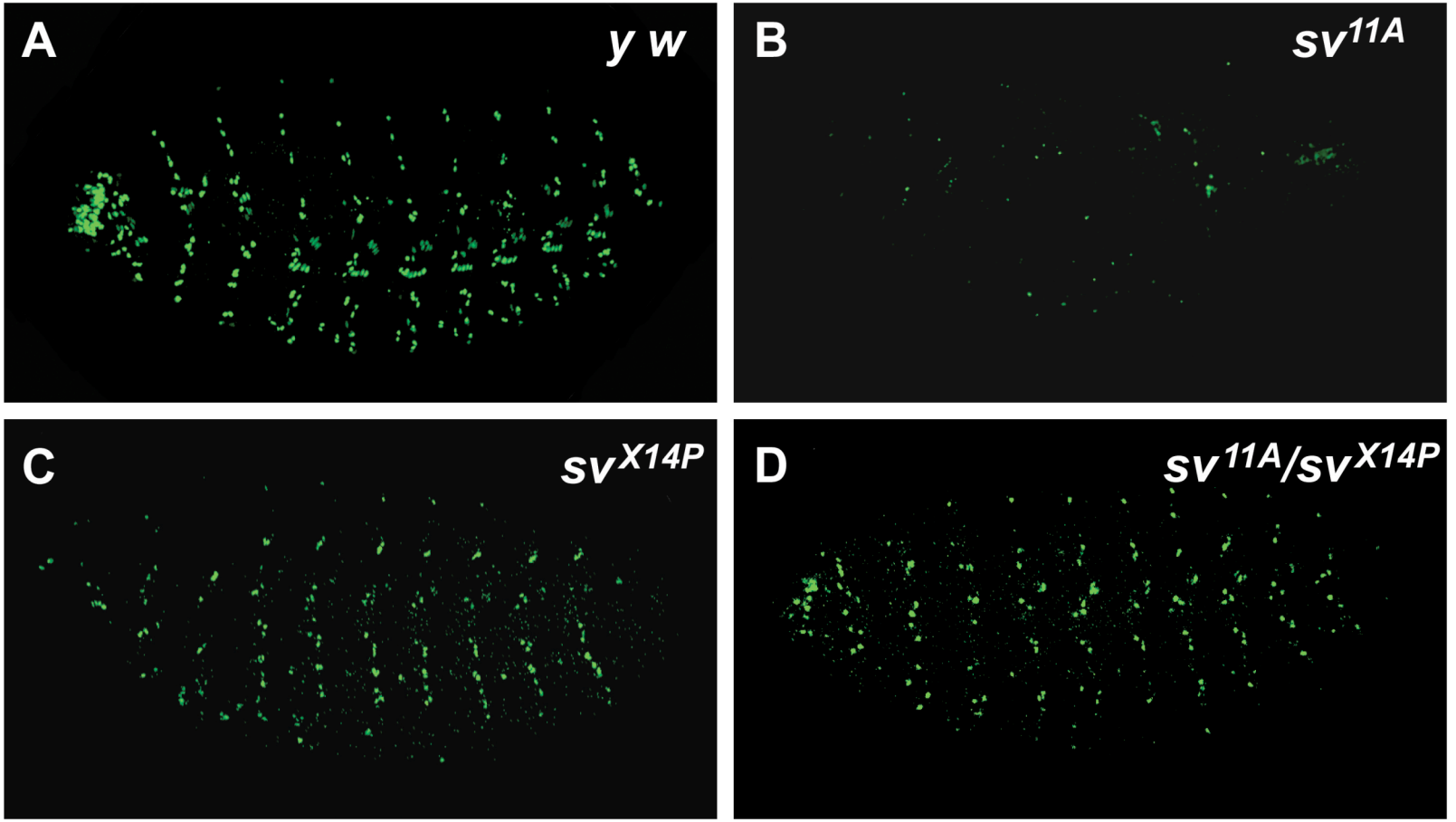

Fig. 9. D-Pax2 expression in embryos.

(A-D) Confocal images of *y w* (A), sv^{11A} (B), sv^{X14P} (C), and sv^{11A}/sv^{X14P} (D) embryos stained with anti-D-Pax2 antiserum are shown. Dorsolateral views of stage 15 embryos are shown.

Chapter 4

The role of *D-Pax2* in the developing CNS of *Drosophila*

Summary

D-Pax2, also known as *sparkling (spa)* or *shaven (sv)*, encodes a paired-domain containing transcription factor. While its function in the developing eye and peripheral nervous system have been described, little attention was payed to its expression in the developing central nervous system. Here we have started to analyze the CNS function of *D-Pax2*. Using generated *D-Pax2* null alleles, we have shown that loss of function in the CNS is lethal. In addition, a conserved enhancer element regulating transcription in the CNS has been mapped to the 6th intron of *D-Pax2*.

Introduction

D-Pax2 encodes a highly conserved paired-domain containing transcription factor closely related to the vertebrate *Pax2*, *Pax5*, and *Pax8* subfamily (Fu and Noll, 1997; Fu et al., 1998). The mammalian *Pax2* gene plays important roles in the development of the ear, eye, kidney, and brain (for review, see Noll, 1993; Dahl et al., 1997). Similarly, *D-Pax2* is required for the proper development of eyes (Fu and Noll, 1997), Johnston's organ, the *Drosophila* ear, and mechanosensory bristles (Fu et al., 1998; Kavaler et al., 1999). Like its mammalian counter part, *D-Pax2* is also expressed in the developing CNS (Fu and Noll, 1997), which suggests a role for *D-Pax2* in CNS development.

The *Drosophila* CNS is a complex organ and serves as an ideal system to study developmental processes such as cell determination, specification, and differentiation (Urbach and Technau, 2004). The *Drosophila* CNS develops from a bilateral neuroectoderm that lies on each side of a narrow strip of ventral midline cells. Neuroectodermal cells delaminate from the surface epithelium and move into the interior of the embryo to form a neural precursor cell, called neuroblast (Campos-Ortega and Hartenstein, 1985; Doe, 1992). A neuroblast divides asymmetrically to give rise to a neuroblast and a ganglion mother cell, which subsequently divides once to produce a differentiating neuron or glial cell. The position of each neuroblast in the developing CNS specifies the type of neurons and glial cells it produces (Chu-LaGraff et al., 1995; Broadus et al., 1995; Bossing et al., 1996a,b). Two classes of genes regulate the selection and delamination of neuroblasts: proneural genes promote neuroblast formation in a proneural cluster of ectodermal cells, one of which is selected fortuitously to become the neuroblast that prevents the remaining cells of the cluster from adopting also a neuroblast fate by the activity of the neurogenic genes, a process called lateral inhibition. The identification of genes controlling the development of the CNS and the characterization of their molecular functions in cell fate determination and differentiation has provided new insights into the problem of how neuronal diversity is generated (Urbach and Technau, 2004).

Here, we have shown that *D-Pax2* plays an important role in the developing CNS, as its CNS function is required for development to adulthood. We have mapped the enhancer of this function, previously shown to activate a separate promoter of *D-Pax2*.

Results

The function of *D-Pax2* in the CNS is vital for development

After the identification of the CNS promoter of *D-Pax2* (Chapter 2), it became possible to study the role of *D-Pax2* in the CNS separately from its other functions that rely on transcription from the PNS promoter governed by the *sv* and *spa* enhancers. To characterize the phenotype of animals lacking *D-Pax2* expression in the CNS, CNS-specific mutant alleles would be highly desirable. Since this could be achieved by the rescue of *D-Pax2* null alleles with the PNS function of *D-Pax2*, we decided to generate such null alleles, again using imprecise excision of the P-element *l(4)2C2*. Five excisions generated null alleles, two of which were characterized in detail. In both null alleles, sv^{12} and sv^{14}, a large region is deleted, extending from the insertion site of the P-element upstream of the PNS promoter to beyond the CNS promoter (Fig.1). The sv^{12} deletion spans 17,726 bp, ending in intron 6. In sv^{14} the deletion extends over 26,974 bp into the 3' flanking region and hence removes more than the entire transcribed region (Fig. 1). Not surprisingly, no *D-Pax2* expression was detected by anti-D-Pax2 immuno-staining in homozygous sv^{12} and sv^{14} embryos (Fig. 2A-C). In addition, sv^{12}/spa^{pol} and sv^{14}/spa^{pol} flies show rough eyes, as expected (Fig. 3). Like $sv^{\Delta 122}$ homozygotes, which lack expression only in the PNS, sv^{12} and sv^{14} larvae have no external mechanosensory organs and die during the first instar. Therefore, up to this lethal phase, additional loss of CNS expression does not result in an observably more severe phenotype. To assess the function of *D-Pax2* in the CNS later in development, we added the *6.7-spa* transgene (Fu et al., 1998) to sv^{12} and sv^{14} homozygotes. This transgene provides the *D-Pax2* PNS function since it was shown to rescue the lethality of animals lacking PNS expression, i.e., of sv^{11A} and $sv^{\Delta 122}$ homozygotes (Shi, 2001). No *6.7-spa*/+; sv^{12} or *6.7-spa*/+; sv^{14} animals survived to adulthood. However live, normal looking second instar and much fewer third instar larvae, but no pupae, were found. We conclude that expression of *D-Pax2* in the CNS is vital for larvae to develop beyond the first instar. In the following we will show that these animals that lack only the CNS functions of *D-Pax2* will also be helpful in the search of transgenes complementing the missing CNS functions.

Genomic region of *D-Pax2* required for CNS function

Previous attempts to find the CNS enhancer of *D-Pax2* tested *lacZ* reporter genes under the control of a CNS promoter, extending 300 bp upstream of the transcription start, for expression in the embryonic CNS. However, transgenes combining various potential

enhancer fragments downstream of the $sv^{\Delta 122}$ deletion (Fig. 1) with this reporter gene showed no embryonic expression (Wälchli, 2003). This negative result can be explained in several ways: (i) the enhancer of interest is not located in the regions tested, (ii) the enhancer was fragmented and thus inactivated by the choice of the regions tested, or (iii) the promoter region selected is not sufficiently large for activation by the CNS enhancer. Homozygous $sv^{\Delta 122\text{-}P}$ embryos exhibit a *D-Pax2* expression pattern in the CNS that is similar, if not identical, to that of wild-type embryos (Fig. 3A,B of Chapter 2). This result excludes the CNS enhancer from the region deleted by $sv^{\Delta 122\text{-}P}$. To determine whether the CNS enhancer is located upstream or downstream of the PNS promoter, two *D-Pax2* alleles, generated by imprecise excision of the P-element of the *l(4)2C2* insertion were very helpful. These alleles, $sv^{26.2}$ and $sv^{51.1}$, delete a large genomic region, encompassing *D-Pax2* and several downstream genes, but not the upstream region spanning the *sv* enhancer and the *PlacW* insertion, which remains at the same location as in *l(4)2C2* (Fig. 1). LacZ expression of *l(4)2C2* embryos suggested that both the *sv* enhancer and the CNS enhancer can activate the P-element promoter driving the *lacZ* gene (Fig. 4A). By contrast, in $sv^{26.2}$ and $sv^{51.1}$ embryos LacZ expression was detectable only in the PNS but not the CNS (Fig. 4B,C), which suggests that the deleted region contains an essential part of the CNS enhancer whereas the upstream region does not contain the CNS enhancer. To further narrow down the region harboring the CNS enhancer, a genomic rescue construct, *D-Pax2 CNS-res*, was prepared. It consisted of a 23 kb genomic fragment that spanned the entire region transcribed in the CNS, extending from the *spa* enhancer to the middle of the neighboring downstream gene, *calsyntenin-1*, which is transcribed in opposite direction (Fig. 1). Two out of three lines were able to rescue the *D-Pax2* CNS null animals to fertile adults. Moreover, D-Pax2 expression in the CNS was rescued, though at reduced levels (Fig. 2D). Therefore, the CNS enhancer is included in the 23 kb of this *D-Pax2* transgene.

A conserved CNS enhancer element in intron 6 of *D-Pax2*

LacZ expression in the CNS of embryos of the *PlacW* enhancer trap line *l(4)2C2* suggested that the CNS enhancer of *D-Pax2* is able to activate *lacZ* under control of the P-element promoter from a position downstream of *lacZ* (Fig. 1). Therefore, we combined a *lacZ* reporter gene (Fig. 6A), similar to *PlacW* (Bier et al., 1989), with *D-Pax2* DNA fragments to be tested for CNS enhancer activity and inserted downstream of *lacZ*. Two such fragments were chosen on the basis of the following considerations. Since functional *cis*-regulatory

elements tend to be conserved between closely related species (Kellis et al., 2003, Chan et al., 2005, Elemento and Tavazoie, 2005), we compared the *D-Pax2* DNA sequence with that of five other *Drosophila* species, using the Genome Vista comparison program (Frazer et al., 2004). The VISTA graph indicates where the conservation between the aligned *D-Pax2* sequences is above 50% (Fig. 5). Regions exhibiting more than 75% of conservation in all *Drosophila* species examined have been considered to harbor conserved functions. These include the coding exons (dark blue regions in Fig. 5) and all known regulatory regions, i.e., the PNS promoter, the *sv* enhancer, and the *spa* enhancer. Interestingly, there is also high conservation at the CNS promoter, which suggests that the use of this CNS-specific transcription initiation site has been conserved in the other *Drosophila* species as well. In addition, a high degree of conservation is observed in sequences of the 6th intron, which thus is a good candidate to include the CNS enhancer. Hence we selected the 2.5 kb of the 6th-intron to be tested for CNS enhancer activity. In addition, the 1.8 kb downstream intergenic region was tested because this region had not been examined in previous attempts to map the CNS enhancer (Wälchli, 2003).

The ability of these regions to drive *lacZ* expression in the CNS was tested by inserting these fragments at a position downstream of *lacZ* into the *PlacW8* vector (Fig. 6A) that was subsequently used for germline transformation. In transgenic embryos, the *lacZ* gene under the control of the 6th-intron was expressed in the CNS in a pattern similar to that of D-Pax2 (cf. Fig. 6B-E with Fig. 2A,D). By contrast, when it was under the control of the downstream intergenic region, it was not expressed in the CNS (Fig. 6F,G). These results suggest that the CNS enhancer is indeed included in the conserved region of the 6th intron.

The enhancer in the 6th intron can drive *D-Pax2* expression in the CNS

If the 6th intron contains the CNS enhancer, it should be able to activate transcription from its cognate promoter, the CNS promoter. To test this, we have generated *D-Pax2-CNS-lacZ* reporter constructs under the control of the 6th intron, which was positioned upstream or downstream of the CNS promoter. Two versions of CNS promoters were used, one extending 760 bp, the other only 300 bp upstream of the start site for CNS-specific transcripts. The unusually large promoter extending to -760 bp was chosen because it includes the entire upstream sequence present in $sv^{\Delta 122\text{-}P}$ embryos (Fig. 1), which display a wild-type D-Pax2 expression pattern in the CNS (Fig. 3D of Chapter 2) and hence must include the CNS promoter. The alternative CNS promoter extending to -300 bp was used

because it had been used in earlier, though unsuccessful, attempts to localize the CNS enhancer. The *D-Pax2-CNS-lacZ* constructs without enhancer served as control. All lines of the transgene under control of the -760 bp promoter and the 6th intron cloned upstream of it showed LacZ expression in the embryonic CNS (Fig. 7B). Double labeling with anti-LacZ and anti-D-Pax2 shows that most cells that express LacZ also express D-Pax2 (Fig. 8). However, the 6th intron did not reproduce the complete CNS expression pattern of D-Pax2 (Fig. 8), which suggest that the 6th intron might not comprise the complete CNS enhancer. When the 6th intron was inserted downstream of *lacZ* in the transgene with the -760 promoter, the LacZ expression pattern in the CNS was similar but much weaker (data not shown). By contrast, *lacZ* transgenes under control of the -300 bp promoter and the 6th intron did not exhibit any detectable LacZ expression in the embryonic CNS (data not shown). These results suggest that the -300 bp CNS promoter region does not comprise the complete promoter, which would explain the failure of previous attempts with *lacZ* reporter genes that included only 300 bp upstream of the CNS transcriptional start site to map the CNS enhancer. The control *D-Pax2-CNS-lacZ* transgenes without the 6th intron did not show any expression of LacZ in the developing embryonic CNS (data not shown). In summary, these results demonstrate that the 6th intron of *D-Pax2* contains an enhancer capable of driving expression in the CNS and that a functional CNS promoter must extend beyond 300 bp, but is included in 760 bp, upstream of the CNS-specific transcriptional start site.

Mapping the CNS-specific transcription initiation site by S1-nuclease

The unusually large CNS promoter raised the question of whether the 5'RACE method did correctly determine the transcriptional start site of this promoter, as an initiation site located further upstream would reduce the size of the CNS promoter. Therefore, it was attempted to determine the initiation site by the independent method of S1-nuclease mapping. As argued above, the true transcriptional start site is less than 760 bp upstream of that determined by 5'RACE. Hence, a region extending from – 760 to + 70 was chosen for S1-nuclease mapping of the transcription initiation site. Four probes of 100 nt length each were selected from this region (P1-P3 and P5 in Fig. 9A), hybridized to total RNA before digestion with S1-nuclease, and the protected 5' labeled probes analyzed on a gel (Fig. 9B). A 70 nt fragment of probe P1, originally extending from -31 to +69, was protected (arrowhead in Fig. 9B). This fragment correlates precisely with the initiation site determined by 5'RACE. In addition, a fragment at 65 bp was observed, which might result from slightly degraded mRNA or an alternative initiation site, located 5 bp downstream. The other three probes,

covering overlapping upstream sequences or a sequence around -760, were not protected against S1-nuclease digestion after hybridization to total RNA (Fig. 9B). Interestingly, all probes showed a weak complete protection, which results from hnRNA transcribed from the PNS promoter that still includes this portion of intron 4. S1-protection experiments with total RNA from *6.7-spa/+; sv*$^{A122\text{-}P}$ embryos and probes P1-P3 showed similar results to those from *y w* embryos (Fig. 9B). However, probe P5 was still present in small amounts as intact fragment (lane 8 in Fig. 9B) and as a much shorter fragment of about 78 nt. This fragment is consistent with the presence of RNA transcribed across the breakpoint of the *sv*$^{A122\text{-}P}$ deletion. In addition, these digests apparently suffered from slightly incomplete S1-digestion, as evident from the presence of small amounts of intact probes. In agreement with these interpretations, control experiments with human RNA did not protect any probe (lanes 9-12 in Fig. 9B). We conclude that there is no initiation site of CNS-specific transcripts different from that previously determined by 5'RACE.

***D-Pax2* transgene driven by the CNS enhancer of intron 6 rescues the lethality of animals that lack D-Pax2 expression in the CNS**

To test whether the lethality of animals lacking D-Pax2 expression in the developing CNS can be rescued by D-Pax2 expression regulated by the CNS enhancer element of intron 6, two CNS-specific *D-Pax2* transgenes were constructed, one with cDNA into exon 9 and genomic DNA downstream of exon 9, the other with a cDNA lacking exons 10 and 11 and genomic DNA downstream of exon 12, both under the control of the CNS promoter and intron 6. The shorter transgene was chosen because the analysis of CNS-specific *D-Pax2* transcripts of wild-type embryos by RT-PCR displayed one most abundant transcript, in which exon 9 is spliced to exon 12 (Fig. 1). Interestingly, both transgenes rescue *6.7-spa/+*; *sv*^{14} animals to adulthood. However, the rescue efficiency of a single copy of each transgene is only about 50%. Since CNS expression of *D-Pax2* in these embryos endowed with only one copy of one of these transgenes was not detectable, it is not clear whether the reduced rescue efficiency results from an incomplete CNS enhancer in intron 6 or from a position effect on the transgene in the lines tested. The rescued flies are weak but did not show any visible morphological defects. Preliminary tests suggest that males and/or females are sterile because stocks could not be established. In conclusion, these results demonstrate that intron 6 contains an important part of the *D-Pax2* CNS enhancer, which may require other elements important for complete rescue of the CNS function.

Discussion

It is known that *D-Pax2* function in the PNS is vital for *Drosophila* development to adulthood (Shi, 2001). In the present study, we have shown that *D-Pax2* also plays a vital role in the development of the CNS. *D-Pax2* loss-of-function in the CNS is lethal during larval stages. At least one copy of the *D-Pax2* CNS gene is necessary for development to adulthood. Previously, the mammalian Pax2 gene has been shown to play a role in CNS development (for review, see Noll, 1993; Dahl et al., 1997). Our study thus seems to imply that, in addition to the PNS and eye functions, *D-Pax2* function in the CNS is conserved from *Drosophila* to humans. Hence, future studies on the function and regulation of *D-Pax2* in CNS development of *Drosophila* might improve our understanding of Pax2 functions in development and diseases of the higher animals.

D-Pax2 is transcribed from two different promoters in a tissue-specific manner. The *sv* enhancer and the *spa* enhancer specifically activate transcription from the PNS promoter, whereas the CNS promoter is specifically activated by the CNS enhancer, which suggests that *D-Pax2* transcription in the CNS is probably regulated by tissue-specific promoter factors (Chapter 2). Tissue-specific use of more than one transcription initiation site has been reported for crystallin genes (van Leen et al., 1986). The recruitment of these tissue-specific promoter-binding factors may direct RNA polymerase II to initiate transcription at their promoter. –Moreover, since the CNS promoter and the CNS-specific first exon are highly conserved among *Drosophila* species, the use of an alternative promoter in the CNS has probably been conserved during evolution.

The genomic rescue construct containing the entire *D-Pax2* CNS gene (Fig. 1) rescues the *D-Pax2* function and expression in the CNS, which suggests that the complete CNS enhancer is confined to this genomic region. By the use of *lacZ* reporter transgenes, we have mapped a conserved CNS enhancer element to the 6th intron of *D-Pax2*. The 6th intron was able to drive *lacZ* expression specifically in the CNS, but it failed to reproduce the complete D-Pax2 expression pattern. Consistent with this observation, a rescue construct expressing D-Pax2 under the control of the CNS promoter and 6th intron was unable to rescue CNS-null animals completely. Therefore, the CNS enhancer in the 6th intron is incomplete and probably requires additional elements for wild-type expression and function. We conclude that the *D-*

Pax2 CNS enhancer consists of more than one DNA segment, probably located in other introns or downstream of the CNS transcription unit.

Materials and methods

Fly stocks

The following fly stocks were used. *y w, ci^{D} spa^{pol}, In(4)spa^{20}* (Fu et al., 1998), *sv$^{\Delta 122}$* (Shi, 2001), *sv$^{\Delta 122\text{-}P}$*, *sv^{12}*, *sv^{14}*, *D-Pax2 CNS-res*, *D-Pax2 CNS-lacZ,* and *l(4)2C2* (kindly provided by J. Kronhamn and A. Rasmuson-Lestander).

S1 nuclease protection assay

For the S1 nuclease protection assay, *D-Pax2* oligonucleotides were obtained from Microsynth and labeled with ^{32}P at their 5'end. 100 μg of total RNA isolated from *Drosophila* embryos (stages 14-17) or from a human embryonic kidney cell line, HEK 293, were used for hybridization, followed by S1 digestion performed as described (Weaver et al., 1979). Total RNA was isolated by TrIzol (Invitrogen) according to the manufacturer's protocol. The following probes were used (positions relative to the CNS transcription initiation site determined by 5'RACE are indicated, and the 10 nt of non-specific sequence added to the 3' end of each probe to monitor S1-nuclease activity are in lower case):

Probe P1 (-31 to +69):
5'CGTTTGTCTACTTCTTTGCATAAGTAAAGTGGACCGAGCACTCTCGTTGCTGTG
GCTTATTTTGTAGGTATGGTCTTCTTTAGTCAAGCCATTGACATTacgcaggcct3'

Probe P2 (-85 to +20):
5'CTGTGGCTTATTTTGTAGGTATGGTCTTCTTTAGTCAAGCCATTGACATTATTTT
TCTTTTATTTAAAAGCGTGTTTTCTCTTTCGTTTTGTGTACCGTAAAATtgatagcaat3'

Probe P3 (-176 to -76):
5'CCGTAAAATATTGCTAACCATTTTTTCCGGACTGTTTGCTGCCAGCCGCAGAGA
TTCTGCGCACGATTTCCTTATTCTTATCTACTTCTACAATTTTTGAGatagggacaa3'

Probe P5 (-793 to -683):
5'CCACAAAGAAGGTCTGTCATCGTGCGCAACGGCACCAATCACAGCACAACTTC
ATGCCTACTTCATTAACGTGTAGCCACTGAGCTTATCGACATCTCAAAgagtgagaac3'

RT-PCR analysis of *D-Pax2* CNS transcripts

Total RNA was extracted from *Drosophila* embryos (stages 14-17) using Nucleo Spin RNA II (MACHEREY NAGEL) kit according to the manufacturer's instructions. cDNA synthesis was carried out with the SuperScript™ II First-Strand cDNA synthesis kit (Invitrogen). PCR reactions were then performed with a pfu polymerase to avoid undesired mutations. PCR cycles are performed with 30 sec denaturation at 94°C, 30 sec annealing at 55°C, and 6 min extension at 72°C. The last cycle is followed by a 10 min incubation at 72°C. The DNA products were analyzed on 1.2% agarose gels. The following primers were used in PCR reactions.

CNS forward: 5'-GTTCCGGCATCGATTACAGGT-3' and

CNS reverse: 5'-GTGTTGCTACTAAAGGTGAACG-3'.

Generation of *D-Pax2* null alleles by P-element imprecise excision

All of over 100 imprecise excisions, except $sv^{\Delta 122}$ (type II in Fig. 10), of the P-element in *l(4)2C2* which do not complement spa^{pol}, also deleted genes downstream of *D-Pax2* (type IV in Fig. 10) and many if not most are terminal deletions of the 4th chromosome. To isolate more type II, and hopefully slightly larger type III deletions, which inactivate the CNS transcription unit of *D-Pax2* (type III in Fig. 10), the screen had to be modified such that no type IV deletions are obtained from the 250 individual F1 crosses. This is the case in the 30 independent F2 *y w; 6.7-spa/+; ex(4)2C2/In(4)*spa^{20} males with rough, spa^{-}, eyes due to type I, II or III excision events since type IV deletions do not survive as they do not complement the lethal inversion breakpoint of $In(4)spa^{20}$ in the downstream *calsyntenin-1* gene (Fig. 10 and Fig. 11, Fu et al., 1998). The transgene *6.7-spa* is needed since the inversion breakpoint upstream of the PNS promoter would be lethal over all mobilization events except clean (type 0) excisions. If the P-element remains in place or excises cleanly (type 0) or the excision creates a deletion towards upstream (type V) the flies have spa^{+} eyes. Interestingly, the *spa* enhancer present in type I excision chromosomes does not activate the PNS promoter of the $In(4)spa^{20}$ chromosome through transvection. This is not that surprising because the *spa* enhancer in $In(4)spa^{20}$ also cannot activate its promoter in *cis* nor in *trans* that of spa^{pol} in $In(4)spa^{20}/ci^{D}$ spa^{pol} flies. Apparently this inversion affects gene activation in *D-Pax-2* from the PNS promoter in many aspects. But for the survival of type III events it was needed that the inversion chromosome provided CNS function of *D-Pax2* if it turns out to be vital. It was impossible to establish these two facts before hand but CNS expression in homozygous embryos and later has a chance to be wild-type. At least it is much closer to wild-type (data

not shown) than PNS expression, which fades away in *In(4)spa*^{20} embryos (Fu, et al., 1998). Because type I deletions retain the *spa* enhancer which complements *spa*^{pol} through transvection (Chapter 3), 17 type I lines could be identified in F3. Although at this stage of the scheme type II deletions are not distinguishable by adult phenotype from type III excision events we were hopeful that the rationale of the screen has worked since 5 type II or III deletions were obtained in F3 as *spa*$^{-}$ flies and the region where the right breakpoint could lie in is much smaller for type II, about 1.9 kb, than for type III deletions, about 15.5 kb. Because the *y w; 6.7-spa/+; ex(4)2C2/ci*^{D} *spa*^{pol} are however not distinguishable by phenotype from their *y w; 6.7-spa/+; In(4)spa*^{20}*/ci*^{D} *spa*^{pol} siblings a stock with the genotype of the other siblings, with the genotype of the F2 father, *y w; 6.7-spa/+; ex(4)2C2/In(4)spa*^{20}, was maintained while the new type II or III chromosomes were isolated from 6 single F3 males from each line. We knew that from this stock *In(4)spa*^{20} can be lost in the case of type II deletions, and possibly also type III deletions, if the CNS function is not vital. Otherwise it is a balanced stock for type III deletions. In the *spa*$^{-}$ *ci*^{D} stocks established from single F3 males without the *6.7-spa* transgene the new type II or III chromosomes were identified and distinguished from *In(4)spa*^{20} on the basis of the absence of the PCR product across the downstream breakpoint of *In(4)spa*^{20} which is not expected to be present in the "simple" type II or III deletion alleles. This worked for all 5 candidates and D-Pax2 expression analysis showed then that 4 type III deletions, among them *sv*^{12} and *sv*^{14}, were obtained since they showed no D-Pax2 expression at all in homozygous embryos. The fifth candidate, *sv*^{20}, is of type II because it shows D-Pax2 expression in the CNS which was not surprising anymore because already a *sv*^{20} homozygous stock with the *6.7-spa* transgene was established which had lost the *ci*^{D} *spa*^{pol} chromosome. The *sv*^{12} allele deletes 17'726 bp starting from the P-element insertion site (Fig. 10 and 1). But 363 bp of the P-element's left end remained at the location of P-element insertion site. The *sv*^{14} allele deletes 26'974 bp, the entire *D-Pax2* transcription unit (Fig. 10 and 1). Here 30 bp of intronic sequence of the third downstream gene, CG-11155, are now at the location of the P-element. It is not known if the deleted DNA has inserted in the 4th chromosome, or elsewhere, without giving rise to detectable D-Pax2 protein, since no Southern blot analysis has been performed.

Plasmid construction and generation of transgenic flies

To rescue the CNS function of *D-Pax2*, the *D-Pax2 CNS-res* construct (Fig. 1) was prepared by cloning the genomic *Kpn*I-*Not*I fragment and the adjacent *Kpn*I fragment from the genomic clones PX11 and PX15 (Fu and Noll, 1997), respectively, sequentially into the

multiple cloning site of the P-element vector pW8. To map the *D-Pax2* CNS enhancer, the *PlacW*-like vector, *PlacW8*, was prepared by cloning the *Hin*dIII-*Eco*RI fragment of the *PlacW* plasmid (obtained from Werner Boll), containing part of the P-element promoter and the *lacZ* gene, between the *Hin*dIII site of the P-element promoter and the *Eco*RI site of the polylinker of the P-element vector pW8. The 6th intron of *D-Pax2* and the downstream intergenic region were amplified by PCR with corresponding restriction sites at their ends and cloned into the multiple cloning site of *PlacW8*. The following oligonucleotides were used as primers in the PCR:
intron 6-forward: 5'-CCGCTCGAGGCTTGGGAGATACGAGATCG-3',
intron 6-reverse: 5'-CGCGGATCCGCTGCTGGTGATGATGTACG-3',
downstream intergenic forward: 5'-CGCGGATCCGTCGCTCATGGTCGTGTAGG-3',
downstream intergenic reverse: 5'-CCGCTCGAGGGAGGAAGTTTCTCCGTCCC-3'.

To test the ability of intron 6 to activate transcription from the CNS promoter, we have generated *D-Pax2-CNS-lacZ* constructs. The *D-Pax2* -300 and -760 promoters and the CNS-specific first exon were amplified by PCR with pfu polymerase and cloned in frame into the *lacZ* containing P-element vector pWZ.1 (Gutjahr et al., 1994). Subsequently, the intron 6 fragment of the *PlacW8*-intron 6 construct was cloned into this construct, upstream or downstream of the CNS promoter-*lacZ* gene. The following primers were used for amplification of the CNS promoter regions:
-300 bp CNS promoter:
forward: 5'-CATTGGCTGCGGCCGCTAAGATGAAAACGCCAACCG-3',
reverse: 5'-ATGGGTACCATTCCATCATGTTCTGGTTGTAGCG-3'.
-760 bp CNS promoter:
forward: 5'-CATTGGCTGCGGCCGCATGTCGATAAGCTCAGTGGC-3',
reverse: 5'-ATGGGTACCATTCCATCATGTTCTGGTTGTAGCG-3'.

For preparation of the CNS-specific *D-Pax2* cDNA in PW8, the *D-Pax2* cDNA was amplified by RT-PCR using total RNA isolated from *Drosophila* embryos (stages 14-17). Sequence analysis of the abundant *D-Pax2* CNS-specific cDNA showed that exon 9 is spliced to exon 12. The cDNA was cloned as *Xba*I-*Pst*I fragment into the pSK^{+} plasmid and its 5' portion extended by a 1.5 kb *Not*I-*Xba*I fragment, taken from *D-Pax2-CNS-lacZ* and containing the -760 CNS promoter and a part of CNS exon 1. The 3' portion downstream of exon 12 was replaced by a 2 kb genomic *Pst*I-*Eco*RI fragment of PCG-2 (Fu et al., 1998).

This whole promoter-cDNA-genomic fragment was then cloned into the P-element vector pW8 and a 0.6 kb *Eco*RI fragment was added to its 3' end in the correct orientation, like in PCG-2. We have also prepared a CNS *D-Pax2* construct where the region between exons 9 and 12 contains genomic DNA by replacing the 919 bp *Spe*I fragment of the 3' portion by the corresponding 3.3 kb *Spe*I fragment from PCG-2. This results in the inclusion of all introns downstream of exon 9. Finally, intron 6 was cloned as 2.5 kb *Xho*I-*Not*I from the *PlacW8*-intron 6 plasmid into both constructs upstream of the *D-Pax2* CNS promoter in its normal orientation.

Immunohistochemistry and microscopy

Embryos were fixed and stained as described (Fu and Noll, 1997). The following antibodies were used: rabbit anti-D-Pax2 antiserum (Fu and Noll, 1997) and rabbit anti-LacZ polyclonal antibody (Cappel) at a 1:2,000 dilution, or chicken polyclonal anti-LacZ antibody (Abcam) at a 1:250 dilution. Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-chicken secondary antibodies were used at a 1:500 dilution (Invitrogen). Biotinylated secondary antibodies against rabbit IgG (Vector Laboratories, Inc.) were used at a 1:300 dilution. For the color reaction, Vectastain ABC Kit (Vector Laboratories, Inc.) was used. Anti-D-Pax2 staining was enhanced by the use of the TSA^{TM} kit (Invitrogen). Microscopy was carried out with a LEICA TCS SP confocal microscope. Images were processed with Adobe Photoshop 7.0 and ImageJ.

Scanning electron microscopy

Scanning electron micrographs of adult fly eyes were taken on a JEOL JSM-6360 LV scanning electron microscope. Female left eyes were pictured at a magnification of 220x.

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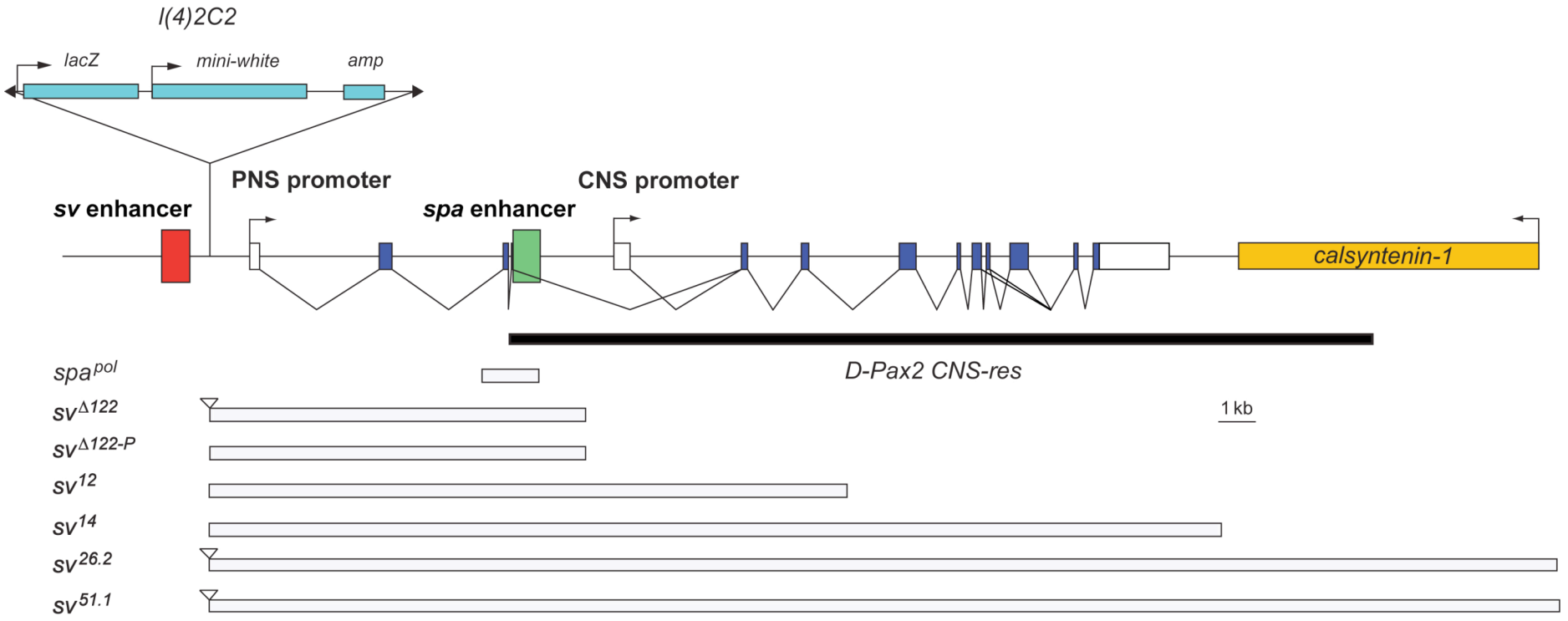

Fig. 1. Molecular and genetic map of the *D-Pax2* locus.

A map of *D-Pax2* and the adjacent downstream gene *calsyntenin-1* (*cals*) (without introns) is shown. The positions of exons (boxed; coding regions in dark blue), of the transcriptional start sites of the PNS and CNS promoters, and of the *sv* (red box) and *spa* (green box) enhancers are indicated. The location of a P-element insertion of the enhancer trap line *l(4)2C2* with a map of its genes (light blue boxes) is shown above. Regions deleted in several deficiency alleles, with the exception of spa^{pol} all generated by imprecise excision of the P-element insertion *l(4)2C2*, are shown below as open boxes with triangles at the left end indicating where the P-element has not been excised but remained in its original position. The black bar below indicates the extent of the genomic fragment used in the CNS rescue construct, *D-Pax2 CNS-res*.

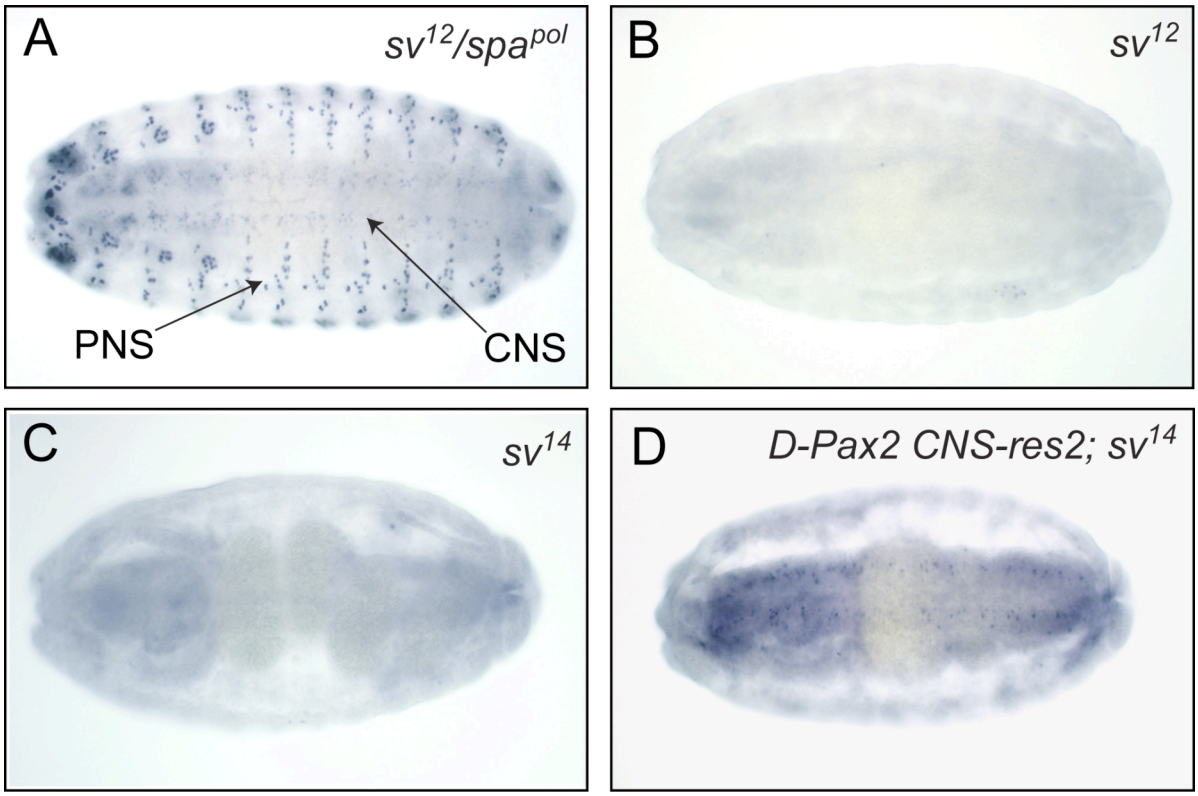

Fig. 2. Absence of D-Pax2 expression in *sv*^{12} and *sv*^{14} embryos and its rescue by a CNS rescue transgene.

(A-D) Expression patterns of D-Pax2 protein are shown in ventral views of stage 15 *sv*^{12}/*ci*^{D} *spa*^{pol} (A), *sv*^{12} (B), *sv*^{14} (C), and *D-Pax2 CNS-res2*; *sv*^{14} (D) embryos.

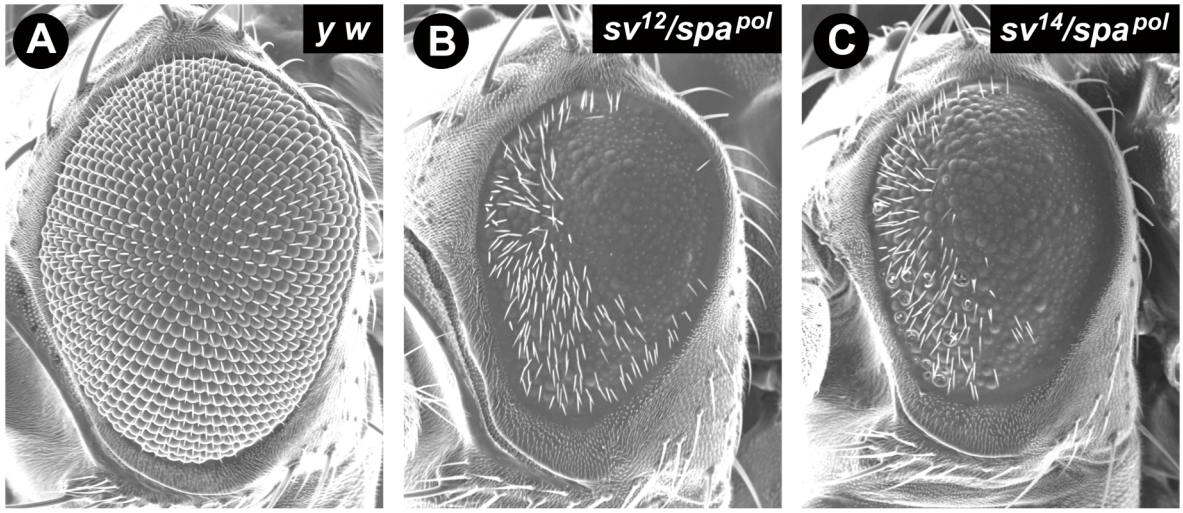

Fig. 3. The *D-Pax2* null alleles do not complement *spa*^{pol}.

(A-C) Scanning electron micrographs of *y w* (A), *sv*^{12}*/spa*^{pol} (B), *sv*^{14}*/spa*^{pol} (C) adult left eyes.

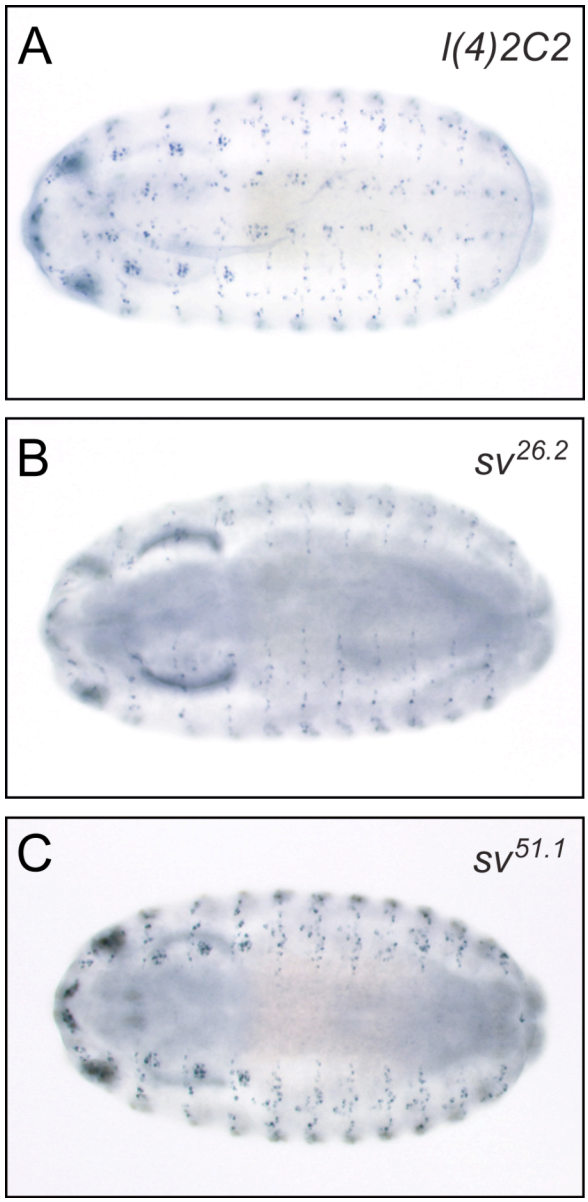

Fig.4. The CNS enhancer is not located in the upstream region of *D-Pax2*.

(A-C) Expression patterns of LacZ in *l(4)2C2/ci*^{D} *spa*^{pol} (A), sv$^{26.2}$ (B), and *sv*$^{51.1}$ (C) embryos. Ventral views of embryos with anterior to the left are shown.

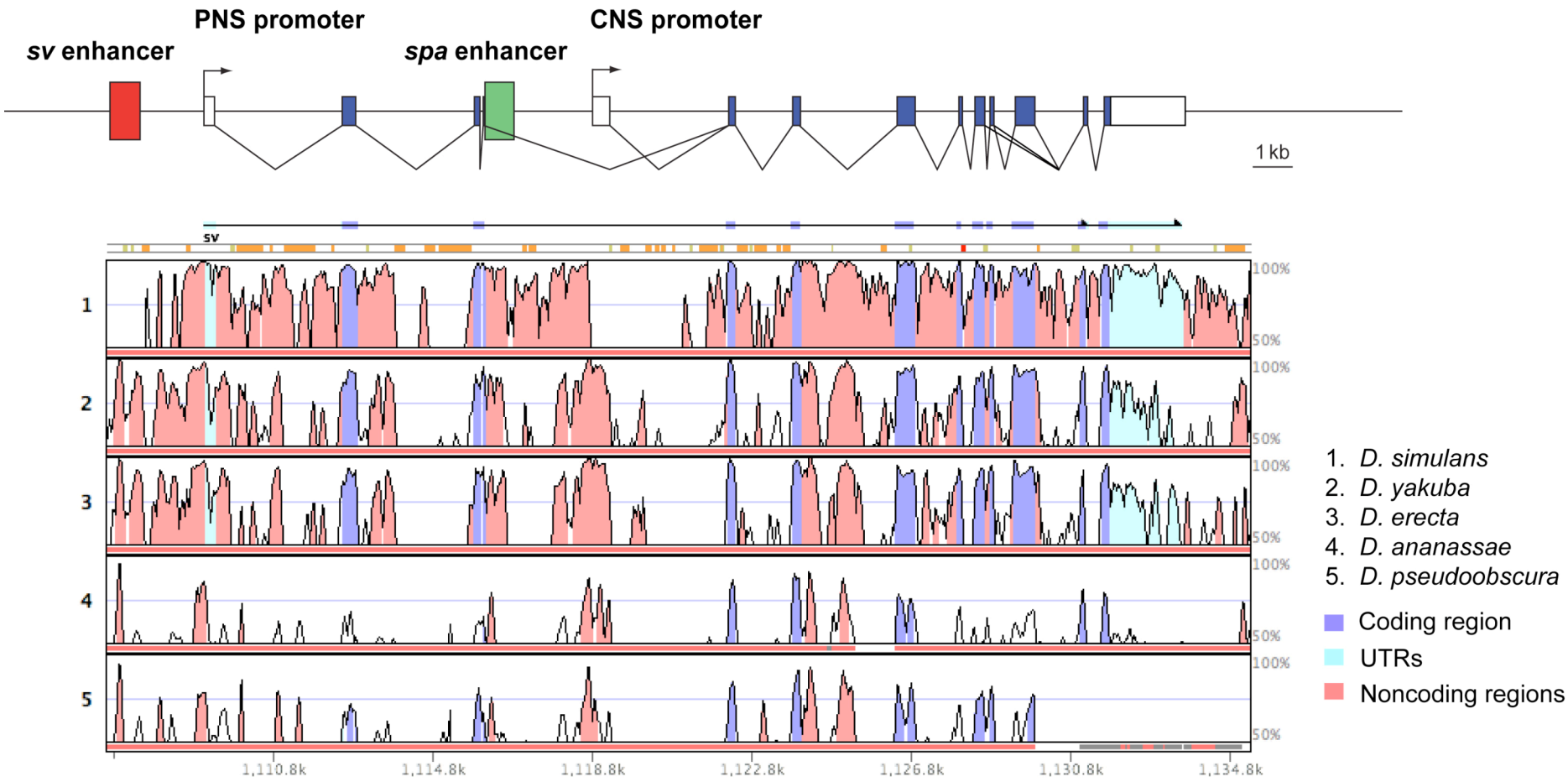

Fig. 5. Vista plot of *D-Pax2* of several *Drosophila* species, illustrating high conservation of DNA sequences.

The relative conservation, above 50% identity in a 100 bp window, of the *D-Pax2* DNA sequence from *Drosophila melanogaster* to that of five other *Drosophila* species (indicated in the right margin) is shown as Vista plot below the corresponding map of the *D-Pax2* gene.

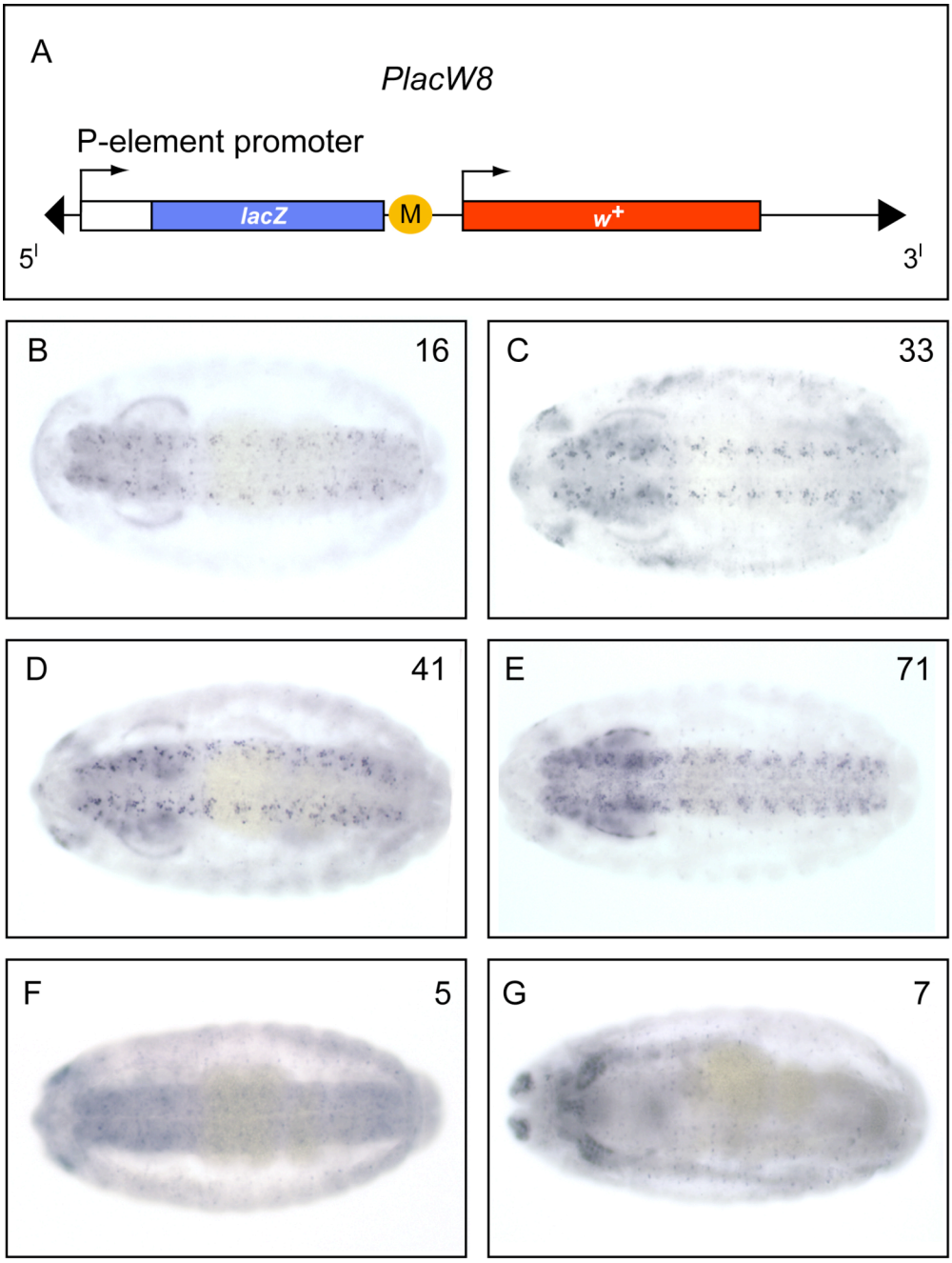

Fig. 6. Embryonic LacZ expression patterns of *PlacW8* reporter genes.

(A) Schematic representation of *PlacW8* vector. **(B-G)** Expression of *lacZ* under the control of *D-Pax2* intron 6 (B-E) or downstream intergenic region (F,G). Ventral views of stage 15 embryos oriented with anterior to the left are shown.

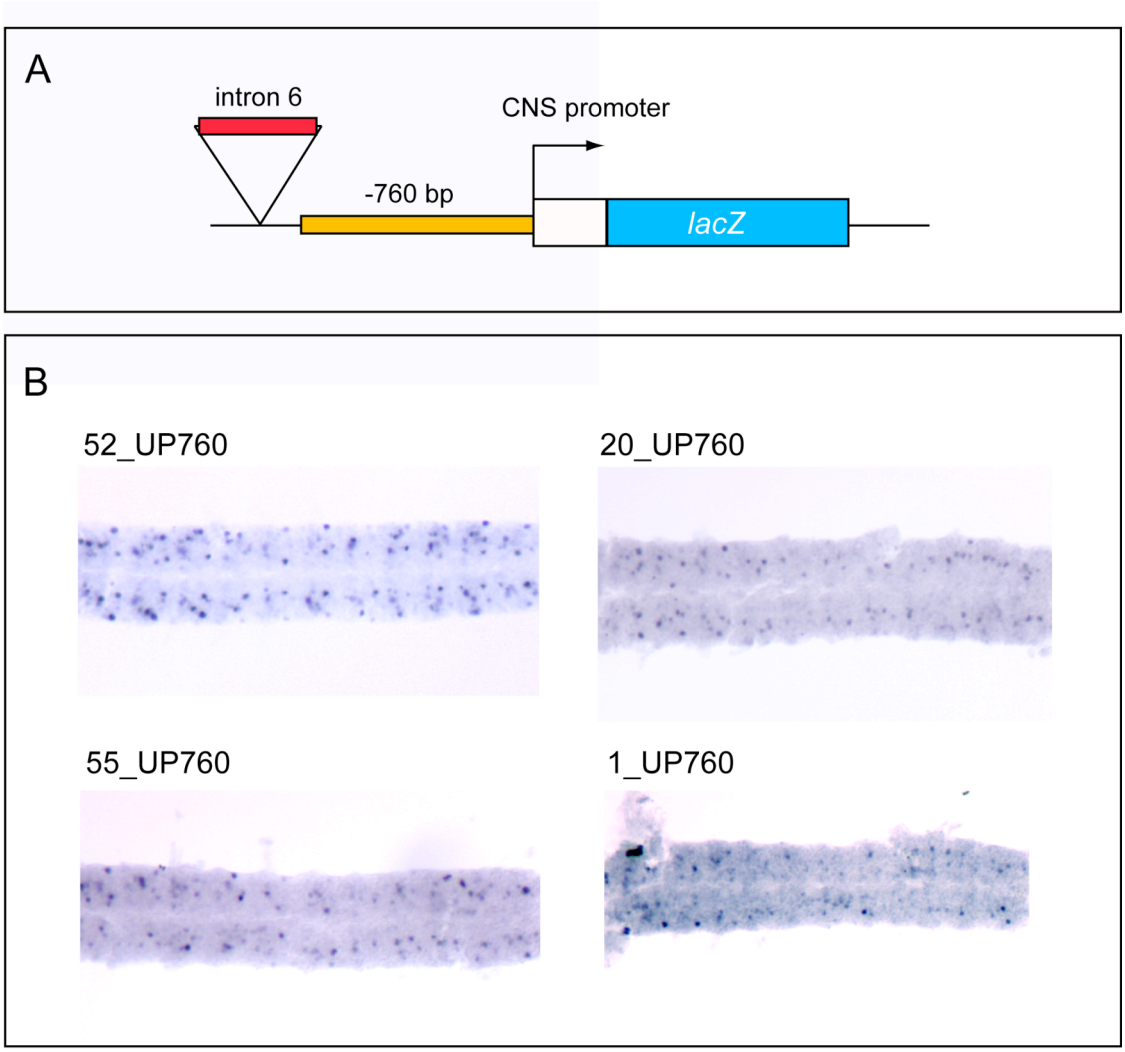

Fig. 7. Intron 6 of *D-Pax2* contains CNS enhancer activity.

(A) Schematic representation of *D-Pax2-CNS-lacZ* reporter construct with intron 6 upstream of the CNS promoter. **(B)** LacZ expressions patterns of four different lines with transgene shown in (A) in ventral nerve chords dissected from stage 15 embryos, oriented with anterior to the left.

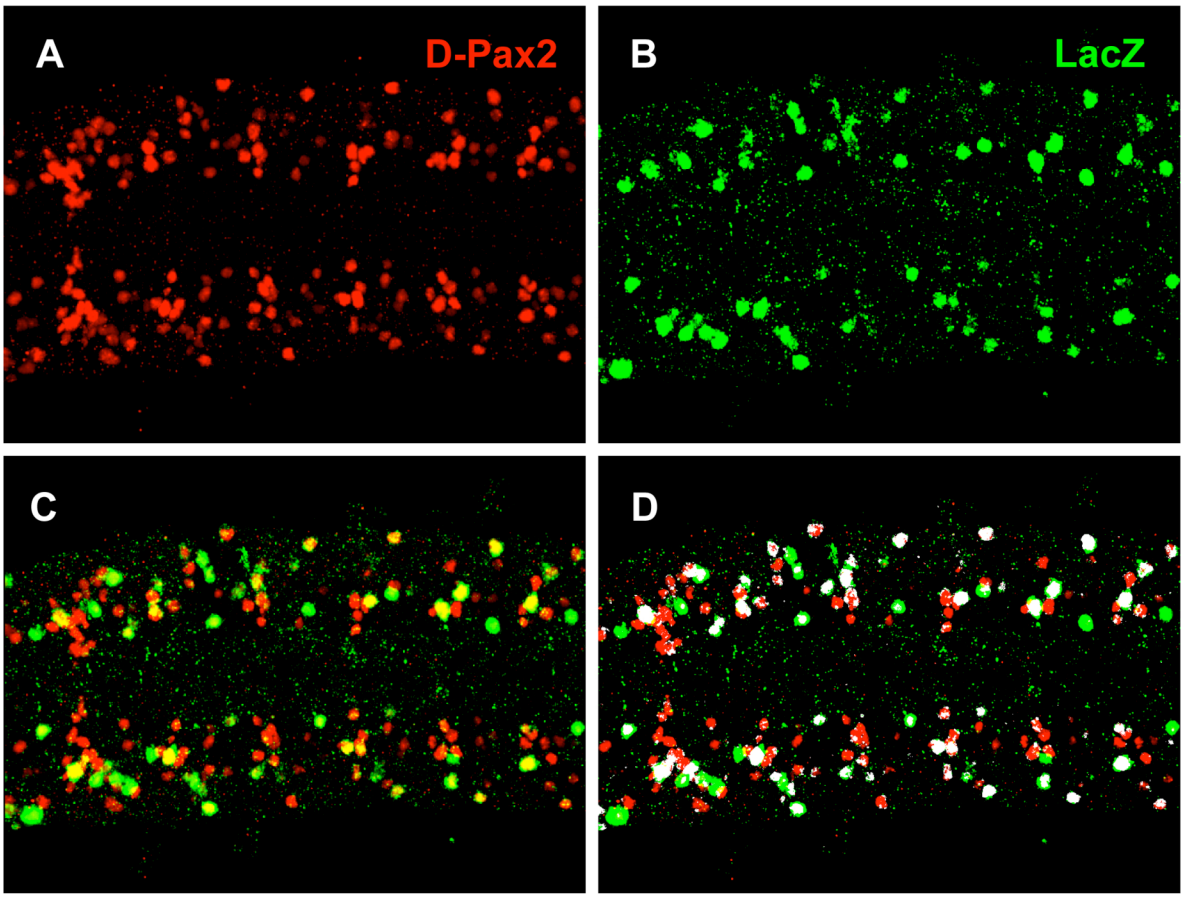

Fig. 8. *D-Pax2* CNS enhancer of intron 6 is incomplete.

(A,B) Confocal images of cells in embryonic CNS expressing D-Pax2 (A) and LacZ (B). Most LacZ expressing cells (green) also express D-Pax2 (red), but not all cells that express D-Pax2 also express LacZ, as evident from the overlay of the two channels **(C)** and ImageJ co-localization **(D)**.

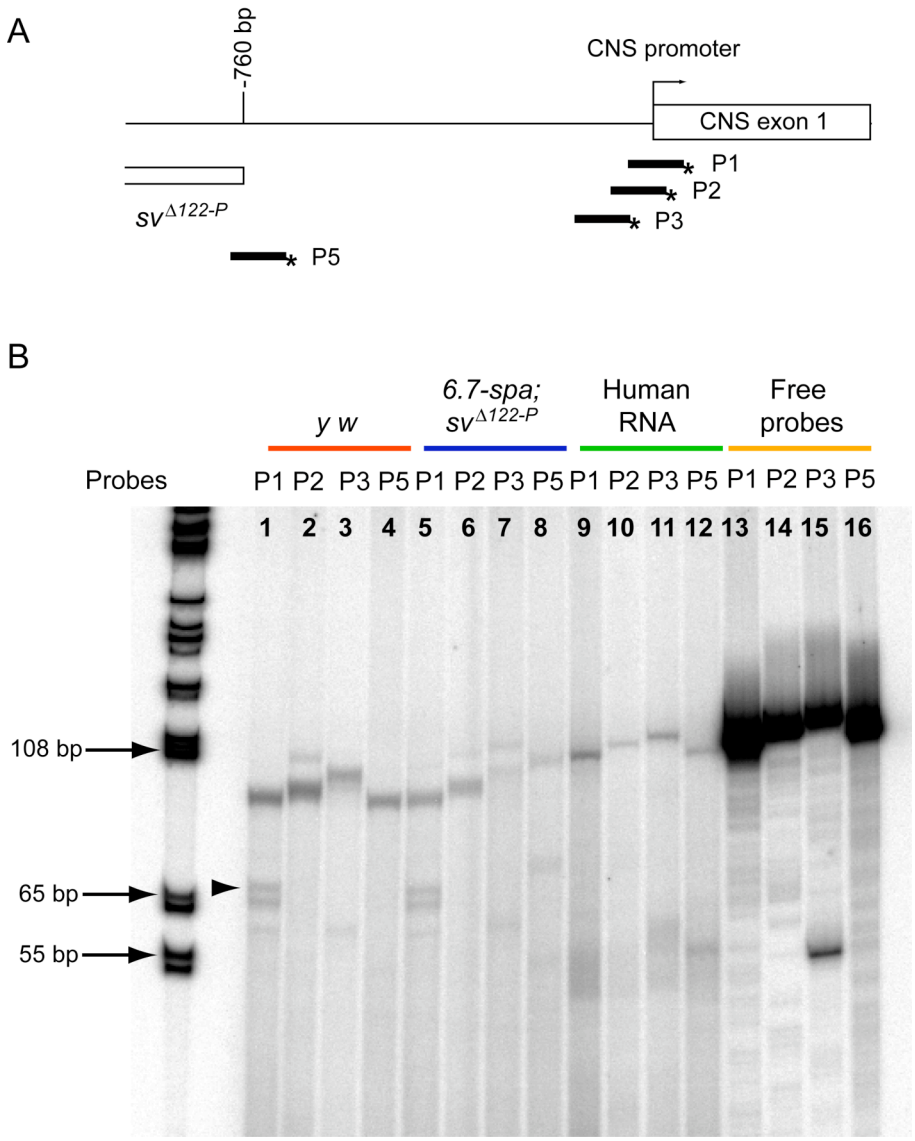

Fig. 9. S1 nuclease mapping of CNS-specific initiation site of *D-Pax2* transcription.

(A) Schematic representation of region including the *D-Pax2* CNS promoter. The horizontal arrow indicates the direction of transcription from the transcription start site determined by 5'RACE. Below, black bars denote the positions of the single-stranded antisense probes (P1-P3 and P5) used for S1 nuclease mapping. The open box on the left represents the 5' end of the region deleted in the $sv^{\Delta 122\text{-}P}$ allele. **(B)** Analysis of protected probes after S1 nuclease digestion by denaturing PAGE. Single-stranded probes P1-P3 and P5, ^{32}P-labeled at their 5' end, were hybridized with total RNA, isolated from the *Drosophila* embryos of indicated genotype or from human HEK cells, digested with S1 nuclease, analyzed by electrophoresis in a 10% polyacrylamide/8 M urea gel, and the protected labeled fragments analyzed by autoradiography. The free labeled probes (diluted 1:100) are shown in lanes 13-16. A marker is shown at the left, and the arrowhead points at the protected band of 70 nucleotides length.

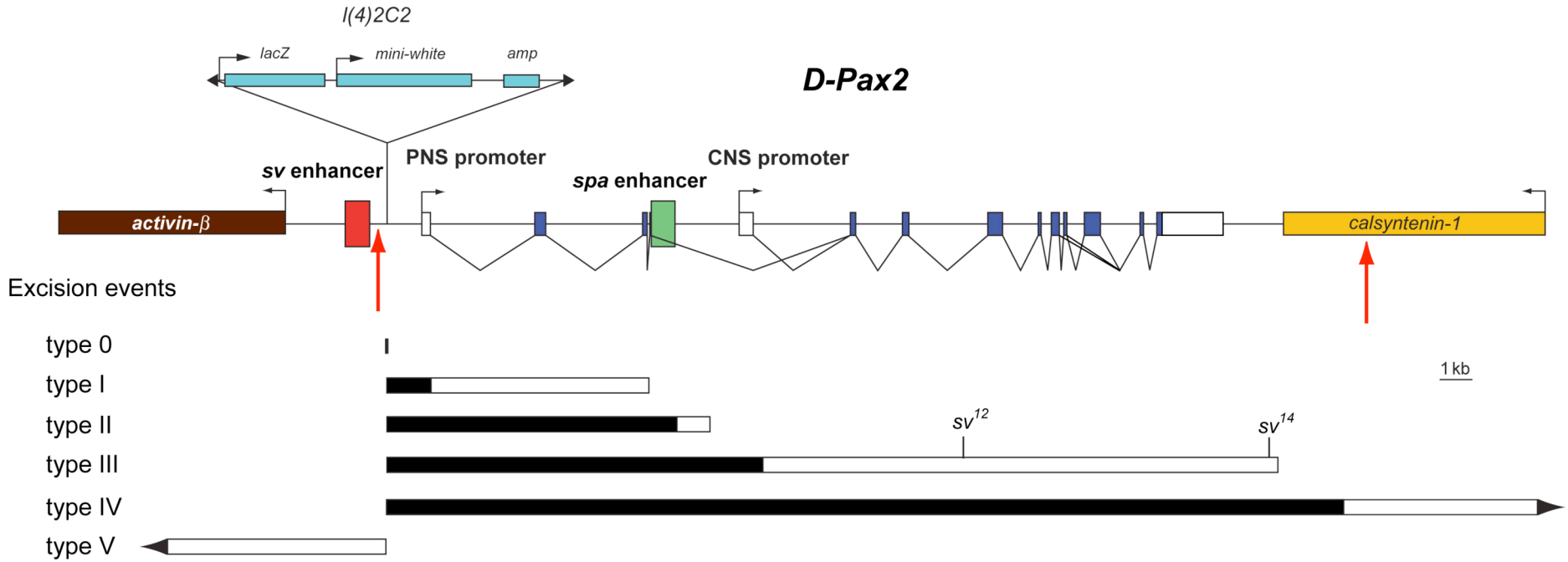

Fig. 10. Illustration of the different imprecise P-element excisions at the *D-Pax2* locus.

A map of *D-Pax2* and the adjacent genes, *activin-β* and *calsyntenin-1* (without introns), is shown. The red vertical arrows below the map indicate the break points of the inversion, *In(4)spa*^{20}. Expected excisions events of the P-element *l(4)2C2*, type 0 to V, are drawn as boxes. The black part of each type indicates the approximate minimum extent of the deletion needed and the white part extends it to the approximate maximum allowed. The arrowheads represent deletions that extend beyond the region shown. The excision events combining type I to IV with type V are not shown.

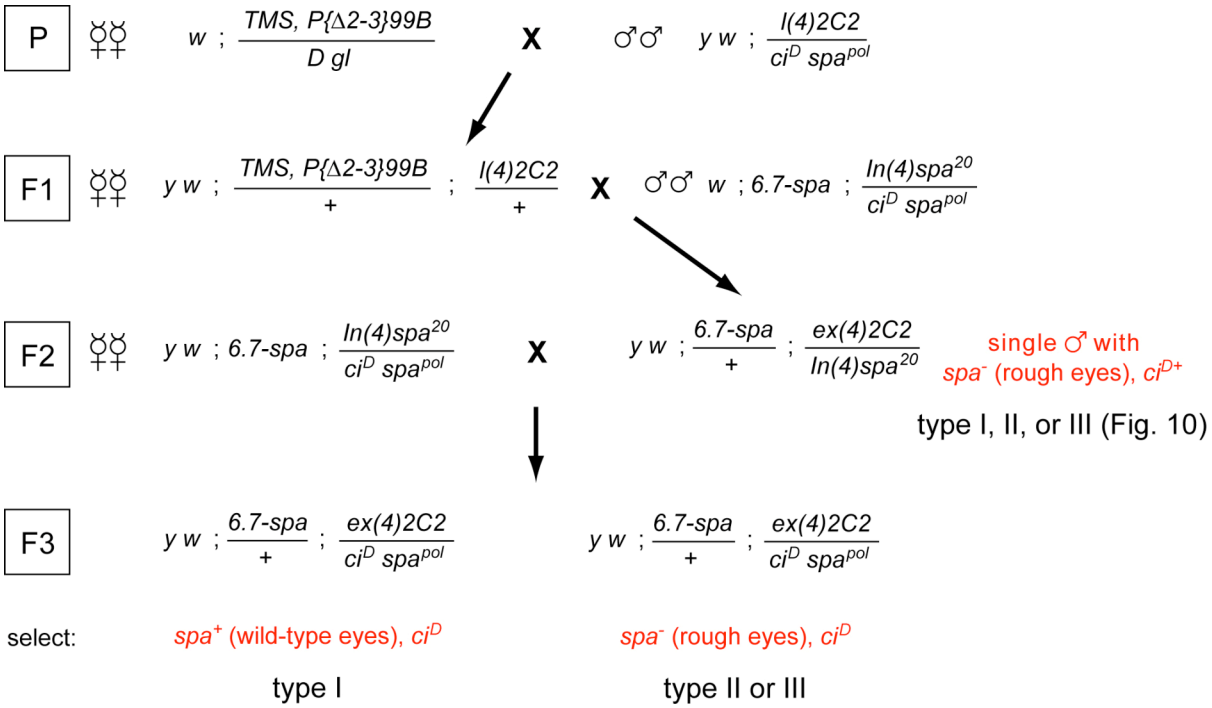

Fig. 11. Crossing scheme for the generation of *D-Pax2* null alleles.

The phenotypes scored for in the offspring of the F1 and F2 crosses are in indicated in red.

Chapter 5

Conclusions and Outlook

D-Pax2, also known as *shaven* (*sv*) or *sparkling* (*spa*), encodes a paired domain containing transcription factor (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999). In *Drosophila* development, it plays important roles in developing eyes and the developing peripheral nervous system (PNS), including the adult mechanosensory bristles (Fu and Noll, 1997; Fu et al., 1998; Kavaler et al., 1999). While its *sv* enhancer regulating transcription in the PNS is located upstream of the PNS promoter (Kavaler et al., 1999; Shi, 2001), the eye-specific *spa* enhancer is included in the 4th-intron (Fu and Noll, 1997). In addition, *D-Pax2* is expressed in the embryonic CNS (Fu and Noll, 1997), where it is transcribed from a different promoter, the CNS promoter (E. Frei, M. Daube, and M. Noll, unpublished). In my thesis I have addressed two important problems concerning the transcriptional regulation of this genetic locus with its two promoters and several tissue-specific enhancers, the specificity between enhancers and promoters and the phenomenon of inter-allelic complementation or transvection.

Enhancer-promoter specificity at the *D-Pax2* locus

The specificity between an enhancer and its cognate promoter has been a fundamental question in gene regulation (Li and Noll, 1994). The mechanisms mediating the enhancer-promoter specificity are not well studied. In this report, we could demonstrate a clear case of enhancer-promoter specificity within a single gene, the *D-Pax2* gene. The *sv* enhancer and the *spa* enhancer specifically activate the PNS promoter but are unable to regulate the CNS promoter, whereas the CNS enhancer can only activate the CNS promoter. Even though the *spa* enhancer is close to the CNS promoter and much closer than to its cognate PNS promoter (Fig. 1 of Chapter 2), it cannot activate the CNS promoter. Similarly, the *sv* enhancer cannot activate the CNS promoter in the PNS, neither in the absence of the PNS promoter when this promoter was deleted, nor when brought by a deletion into close proximity of the CNS promoter. These experiments have excluded the alternative models of promoter competition and insulator DNA elements mediating the enhancer-promoter specificity at this locus. Thus,

the enhancer-promoter specificity at the *D-Pax2* locus can only be explained by the compatibility between the enhancers and their cognate promoters, as shown previously for other genes (Li and Noll, 1994; Merli et al., 1996). Experiments with the more promiscuous P-element promoter further suggested that the promoters rather than the enhancers control the specificity between enhancers and their promoters because both the *sv* enhancer and the CNS enhancer, which exclusively activate their cognate promoters, can activate the P-element promoter. Therefore, characterizing the regulatory sequences of these promoters and the protein factors interacting with them is expected to shed light on the molecular mechanism regulating the compatibility between enhancers and promoters and thus transcriptional specificity.

Transvection at the *D-Pax2* locus

Ed Lewis, discovered the genetic phenomenon of pairing-dependent inter-allelic complementation at the *Ultrabithorax* (*Ubx*) locus of *Drosophila* and called it transvection (Lewis, 1954). Its molecular basis turned out to be the ability of an enhancer to activate transcription from its cognate promoter located on the paired homologous chromosome (Morris et al., 1998; Morris et al., 1999). We found *D-Pax2* to be a novel locus with the property of transvection. The complementation of the *spa* enhancer deficiency, spa^{pol}, with the promoter deletion allele, sv^{11A}, produced a nearly wild-type eye that contrasts the strong rough eye phenotype of spa^{pol} flies. Since the *spa* enhancer can act only through the PNS promoter, this complementation of spa^{pol} with sv^{11A} in the eye can be explained only by transvection, i.e., by the activation of *D-Pax2* transcription through the intact *spa* enhancer of the sv^{11A} allele acting on the intact PNS promoter of the spa^{pol} allele in the developing eye imaginal disc. This conclusion was corroborated by direct identification of the *D-Pax2* transcripts produced in the eye disc by RT-PCR. Only shortened transcripts originating from the spa^{pol} chromosome and no wild-type *D-Pax2* transcripts were found. Interestingly, the *spa* enhancer was able to activate transcription from the promoter in *cis* and as well as in *trans*. In contrast to the *spa* enhancer, the *sv* enhancer does not support transvection. The *sv* enhancer deletion allele, sv^{X14P}, did not complement the promoter deletion allele, sv^{11A}. Analysis of *D-Pax2* transcripts by qRT-PCR showed that the *sv* enhancer is able to activate transcription minimally in *trans*, at a level that is not sufficient for complementation. These results strongly suggest that transvection is a property of the enhancer, but not of the promoter, as of two different enhancers activating the same promoter in *cis* only one can

activate it in *trans* at a level sufficient to complement the lacking function. In addition, this property has a quantitative aspect, as evident from a slight suppression of the strong lack-of-function phenotype by the *sv* enhancer acting in *trans*. Thus, transvection at the *D-Pax2* locus depends on the properties of the individual enhancers. As Erich Frei in our lab aptly coined it: "Not every "*cis-enhancer*" is also a "*trans-enhancer*".

These contrasting properties of *D-Pax2* enhancers to support transvection provide an ideal system for future studies investigating the molecular mechanisms of transvection. In addition, it will be interesting to test the *D-Pax2* enhancers for transvection at ectopic loci of this gene, (i) to test whether transvection of *D-Pax2* depends on the pairing of the alleles, the original test of Ed Lewis, and (ii) to test whether the results obtained at the *D-Pax2* locus can be reproduced in a different chromosomal environment. It is possible to test this by using the PhiC31 integrase system (Bischof et al., 2007). Preliminary experiments confirm our conclusions.

***D-Pax2* function in the CNS**

We have generated *D-Pax2* null alleles, sv^{12} and sv^{14}, that contain deletions uncovering the PNS as well as the CNS transcription units (Chapter 4, Fig.1). Homozygous sv^{12} and sv^{14} embryos show complete loss of D-Pax2 expression in the PNS and CNS and die as first instar larvae. These animals cannot be rescued to adults by merely providing the PNS function of *D-Pax2*. Therefore, *D-Pax2* function in the CNS is also vital for development to adulthood. To understand the molecular role of *D-Pax2* in the *Drosophila* CNS development, however, a detailed analysis of its expression at the cellular level during development is indispensable. Genetic experiments, confirmed by 5′ RACE, indicated that transcription in the CNS starts from a second promoter of *D-Pax2*, the CNS promoter. This transcription initiation site was further corroborated by S1 nuclease mapping. Since the genomic transgene, D-Pax2 *CNS-res*, containing the complete *D-Pax2* CNS transcription unit, was able to rescue expression and function of *D-Pax2* in the CNS, the CNS enhancer could be localized to this region of the *D-Pax2* gene. Finally, we have found a conserved element in intron 6 that is able to drive *lacZ* expression in the CNS similar to *D-Pax2*. Intron 6 comprises a crucial, but not complete, CNS enhancer since CNS-specific *D-Pax2* cDNA under the control of intron 6 and the CNS promoter is able to rescue the lethality of CNS loss-of-function animals to about 50% viability. We speculate that additional CNS enhancer elements are required for complete rescue. Thus, it appears that the CNS enhancer is a complex enhancer consisting of

more than one region separated by exons, as we have encountered it for enhancers of the other *Pax2*-like gene of *Drosophila*, *Pox neuro* (Boll and Noll, 2002).

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Curriculum Vitae

Surname:	Chilaka
First name	Sabarinadh
Date of birth	April 13th 1979
Place of birth	Kandukur, Andhrapradesh, India
Nationality	India
Address	Glaubtenstrasse 42, 8046 Zurich, Switzerland

Education

1994-1996	Board of Intermediate Education, Andhra Pradesh, India. Botany, Chemistry, Physics and Zoology
1996-1999	Bachelor student of Nagarjuna University, India Specialized in Botany, Chemistry and Zoology Degree: Bachelor of Science
1999-2001	Master student of Pondicherry University, India. Specialized in Biotechnology Title of the thesis: Development of PCR based Diagnostic Kit and Outer Membrane Protein (OMP) Vaccine against *Aeromonas hydrophila* in *Cyprinus carpio.* Supervisor: Dr. V. Arul Degree: Master of Science
2003-2008	PhD student at Institute of Molecular Biology, University of Zurich, Zurich, Switzerland. Title of the thesis: Transvection and Enhancer-Promoter specificity at the *D-Pax2* locus during *Drosophila* development Supervisor: Prof. Dr. Markus Noll

Work experience

2002-2003	Junior research fellow in the lab of Dr. Rakesh Mishra at Center for Cellular and Molecular Biology (CCMB), Hyderabad, India.

Publications

Sabarinadh, C., Subramanian, S., Tripathi, A. and Mishra, R. K. (2004). Extreme conservation of noncoding DNA near HoxD complex of vertebrates. *BMC Genomics* 5, 75.